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THE BIOSYNTHESIS AND CONTROL OF INDOLEACETIC ACID

by

Robert Malcolm Simpson

This thesis is presented in fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry at the University of Auckland.

ABSTRACT

Attempts were made to form indoleacetic acid in cellfree extracts of mung bean (Vigna radiata) shoots. The extracts were incubated with radiolabelled tryptophan and other substrates and cofactors thought to be involved in indoleacetic acid biosynthesis. After incubation indolepyruvate and indoleacetic acid were separated and quantified by HPLC. There was no significant difference in the conversion of tryptophan to indolepyruvate and indoleacetic acid between the incubations and control incubations using boiled extract.

The concentrations of indolepyruvate and indoleacetic acid in mung bean hypocotyl suspension cultures were measured using GC-MS SIM over the growth of the culture, a period of 29 days. Indoleacetic acid concentrations, although scattered, mostly remained at constant low levels in the range of 6 to 9ng/g fwt of culture. The indolepyruvate levels steadily increased to a maximum level after 14 days, then remained at this level, 10 to 12 ng/g fwt, for the remainder of the culture period. This plateau in indolepyruvate concentration matched the period that the suspension culture was in the logarithmic phase of growth.

An aromatic amino acid aminotransferase was purified over 33,000 fold from the shoots and primary leaves of mung beans, as determined using a tryptophan aminotransferase activity assay. The enzyme was a monomer, with a molecular weight of about 58kDa. The pH optimum was broad, with a maximum at about 8.6. The relative activities of the aromatic amino acids were: tryptophan 100, tyrosine 83 and phenylalanine 75, and the K_m's were 0.095, 0.08 and 0.07mM respectively. The enzyme was able to use 2-oxoglutarate, oxaloacetate
and pyruvate as the oxo acid substrate at relative activities 100, 128 and 116 and $K_m$'s 0.65, 0.25 and 0.24mM respectively.

In addition to the aromatic amino acids the enzyme was able to transaminate alanine, arginine, leucine and lysine to a lesser extent, and showed slight activity with asparagine, aspartate, histidine, valine and D-tryptophan and tyrosine. Inhibition studies showed that the alanine, aspartate and histidine activities were part of the aromatic amino acid aminotransferase activity.

The enzyme was not inhibited by indoleacetic acid, although naphthaleneacetic acid did inhibit slightly. There was evidence of substrate inhibition by hydroxyphenylpyruvate at high concentrations. Addition of the cofactor pyridoxal phosphate only slightly increased the activity of the enzyme.

The enzyme was blotted onto a PVDF membrane cleaved by in situ trypsin digest. Three of the tryptic fragments were sequenced. These fragments had approximately 60% sequence similarity with plant aspartate aminotransferases and tyrosine aminotransferases.
ACKNOWLEDGEMENTS

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<td>Mass spectroscopy</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NAA</td>
<td>Naphthalene-2-acetic acid</td>
</tr>
<tr>
<td>NAD\textsuperscript{+}</td>
<td>(\beta)-Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>(\beta)-Nicotinamide adenine dinucleotide, reduced form</td>
</tr>
<tr>
<td>NADP\textsuperscript{+}</td>
<td>(\beta)-Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>(\beta)-Nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>OAA</td>
<td>Oxalacetate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymer chain reaction</td>
</tr>
<tr>
<td>PFB</td>
<td>Pentafluorobenzyl</td>
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1 INTRODUCTION

There is a large body of information on the various responses of plants to applied indole-3-acetic acid (IAA). Indeed the effect of indoleacetic acid on shoot growth was observed by Darwin (1880), well before isolation of the auxin, and was used to postulate its existence. In contrast, details of its biosynthesis, metabolism, regulation and the relationship between endogenous levels and the plant response are largely unknown.

1.1 Auxins

There are a number of groups of substances, produced in low concentrations that regulate physiological functions in plants. These are known as phytohormones or plant growth regulators and have been divided into five major classes according to structure: abscisic acid, ethylene, gibberellins, auxins and cytokinins, although other compounds exist which may be plant growth regulators (e.g. polyamines and brassinosteroids). Each class is able to elicit a wide range of responses depending on the species being tested, what tissue or organ it is acting upon, the age of the plant, the concentration of the plant growth regulator and what other growth regulators are present. For example, the same concentration of indoleacetic acid which causes shoot growth and development in beans inhibits the growth of roots (Cleland, 1988); yet auxin in lower concentration stimulates the development of roots in many plant species.
Indoleacetic acid (Fig. 1.1a) is the best known and most widely studied natural auxin. It appears to be ubiquitous in the plant kingdom, being found in seed plants, fungi and algae as well as many bacteria (Audus, 1972). There are a number of other endogenous substances which show auxin activity including 4-chloroindoleacetic acid (Engvild et al., 1978; Fig. 1.1b) and phenylacetic acid (Wightman and Lighty, 1982; Fig. 1.1c). A range of synthetic auxins have also been discovered, including naphthaleneacetic acid (Fig. 1.1d), 2,4-dichlorophenoxyacetic acid (2,4-D; Fig. 1.1e) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T; Fig. 1.1f). These have important roles in agriculture as herbicides and promoters of fruit set and crop development. As well as existing in the plant as the free compound, indoleacetic acid is also found conjugated to amino acids and sugars. The most common of these are indole-3-acetyl-1-O-glucose, indole-3-acetyl-L-aspartate, indole-3-acetyl-2-O-myoinositol, indole-3-acetyl-2-O-myoinositol-arabinoside and indole-3-acetyl-2-O-myoinositol-galactoside (Cohen and Bandurski, 1982; Figs 1.2a-1.2e).

Auxins induce many responses in plants. At the cellular level they induce cell elongation in shoot apical meristems, the classical auxin response; they also stimulate cambial cell division (Reinders-Gouwendak, 1965), initiate root growth (Burstrom, 1969; Gaspar, 1972; Krikorian et al., 1987) and stimulate differentiation of phloem and xylem (Jacobs, 1952). At organ and whole plant level they maintain apical dominance: auxin from the apical bud represses the lateral buds (White et al., 1975). Auxins inhibit leaf abscission and delay leaf senescence, probably in conjunction with their regulatory effects on ethylene biosynthesis (Aharoni et al., 1979). Auxins delay fruit ripening and allow development of parthenogenic fruit (Luckwill, 1953; Ludford, 1987). They promote the growth of floral organs and promote flowering.
Fig. 1.1. Structures of Natural and Synthetic Auxins.

Natural Auxins:  (a) Indoleacetic acid  
                 (b) 4-Chloroindoleacetic acid  
                 (c) Phenylacetic acid  

Synthetic Auxins: (d) Naphthaleneacetic acid  
                  (e) 2,4-Dichlorophenoxyacetic acid  
                  (f) 2,4,5-Trichlorophenoxyacetic acid
(a) Indoleacetic Acid

(b) 4-Chloroindoleacetic Acid

(c) Phenylacetic Acid

(d) Naphthaleneacetic Acid

(e) 2,4-Dichlorophenoxyacetic Acid

(f) 2,4,5-Trichlorophenoxyacetic Acid
Fig. 1.2. Structure of Natural Auxin Conjugates.

(a) Indole-3-acetyl-1-O-glucose.
(b) Indole-3-acetyl-L-aspartate.
(c) Indole-3-acetyl-2-O-<i>myo</i>-inositol.
(d) Indole-3-acetyl-2-O-<i>myo</i>-inositol-arabinoside.
(e) Indole-3-acetyl-2-O-<i>myo</i>-inositol-galactoside.
<table>
<thead>
<tr>
<th>Plant species</th>
<th>Organ/Growth phase</th>
<th>Free IAA ng/g</th>
<th>Conjugated IAA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Avena sativa</em></td>
<td>shoot</td>
<td>16</td>
<td>5</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>seed</td>
<td>440</td>
<td>7620</td>
<td>nd</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
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<td>24</td>
<td>328</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>root-stele</td>
<td>68</td>
<td>64</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>-cortex</td>
<td>13</td>
<td>61</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>seed</td>
<td>500</td>
<td>66</td>
<td>-</td>
</tr>
<tr>
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<td>37</td>
<td>473</td>
<td>76</td>
</tr>
<tr>
<td><em>Lycopersicum esculentum</em></td>
<td>shoot</td>
<td>7.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>Cambium:</td>
<td>250</td>
<td>20</td>
<td>22</td>
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<tr>
<td></td>
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<td>250</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>mid growth</td>
<td>460</td>
<td>28</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>end growth</td>
<td>480</td>
<td>28</td>
<td>24</td>
</tr>
<tr>
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<td>25</td>
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<td>nd</td>
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<tr>
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<td>shoot</td>
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<td>nd</td>
<td>nd</td>
</tr>
<tr>
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<td>5</td>
<td>43</td>
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<tr>
<td></td>
<td>seed</td>
<td>93</td>
<td>nd</td>
<td>202</td>
</tr>
</tbody>
</table>

Table 1.1 Levels of Free and Conjugated Indoleacetic Acid *In Vivo*.  
nd = not determined; - = below detectable levels
in some species (Bernier et al., 1981). It is also possible that auxins mediate the tropistic responses to light and gravity, although the endogenous role is not certain (Kaufman and Song, 1987).

Endogenous indoleacetic acid concentration varies from species to species, as does the amount of bound versus free indoleacetic acid (Table 1.1). In vegetative tissue the concentration of free indoleacetic acid is often found within a range of 10 to 30 nanograms per gram fresh weight, but in some tissues such as seeds, the levels of both free and bound indoleacetic acid are particularly high (Table 1.1).

1.1.1 Primary Mechanisms of Auxin Action

The mechanism of auxin action, especially with regard to stimulation of growth, is poorly understood. Temporal changes in effect of exogenous auxin on growth rates are biphasic: after a lag of 7-10 minutes the growth rate rises rapidly to a peak, then falls to the minimum, before rising to a sustained steady state rate, slightly lower than the first maximum, after about an hour after exposure to the auxin (Barkley and Evens, 1970; Bouchet et al., 1983). Several minutes after the auxin stimulation and prior to any visual effects there is an induction of new mRNA species (Hagen, 1987), due to changes in mRNA transcription. Additionally, the use of inhibitors of protein synthesis have shown that protein synthesis is required for any of the growth inducing effects of auxin (Bates and Cleland, 1979).

Several models have been proposed to explain how auxin affects the growth rate. None of these models alone is able to explain all of the observations and it is likely that the true situation is a composite of
several models occurring at the same time, causing various portions of the effects of auxin.

The first model, the acid growth theory (Vanderhoef and Dute, 1981) proposed that auxin regulates two processes: wall loosening, breaking of various bonds within the polysaccharide cell wall to allow expansion of the cell by turgour pressure, (first response) and wall synthesis (second response). Wall loosening is suggested to result from H+ extrusion and lowered cell wall pH, probably caused by the activation of a plasma membrane-bound ATPase. This acidification could then activate polysaccharide-degrading cell wall enzymes (Cleland and Rayle, 1978). The sustained growth is dependent on continued cell wall and protein synthesis (Guilfoyle, 1986; Hagen et al., 1984).

The acid growth theory has been critically examined by Kutschera and Schopfer (1985a, 1985b) and Luthen and coworkers (1990). The former showed that a fungal toxin, fusicoccin, which induces proton secretion caused growth similar to that of exogenous auxin, although they found the effects of auxin and fusicoccin to act via different mechanisms, suggesting that the theory was inadequate. However, Luthen and coworkers (1990), repeating the experiments under more rigorous conditions showed that the effects of fusicoccin and auxin were similar: if the two substances were applied one after the other the resulting effects were not additive.

Thus it is nearly certain that growth occurs at least partly by the acid growth theory. Yet this theory does not explain a number of other requirements and observations of auxin induced growth: the need for protein synthesis, the lag period and the induction of mRNA. Thus, other features must also operate.

A second model (Theologis, 1981) involves greater use of second messengers. Indoleacetic acid binds to a plasma membrane receptor,
which mediates the release of $\text{Ca}^{2+}$ from the endoplasmic reticulum and/or the vacuole via an undetermined second messenger, probably some kind of phosphoinositide. The rise in cytosolic $\text{Ca}^{2+}$ activates $\text{Ca}^{2+}/\text{H}^+$ antiports and $\text{Ca}^{2+}$/ATPases, returning $\text{Ca}^{2+}$ levels to normal. The $\text{Ca}^{2+}$ sequestration leads to lowered cytosolic pH which results in loosening and synthesis of cell walls by the manner described in the acid growth theory. However the increased $\text{Ca}^{2+}$ level, possibly via calmodulin, stimulates the activity of protein kinases and hence brings about protein phosphorylation. This could activate a protein already present in the cytosol so that it is capable of binding indoleacetic acid. The protein-indoleacetic acid complex then moves into the nucleus, where it interacts to activate the transcription of specific mRNA sequences, including polysaccharide synthases, thus causing the second phase of growth.

Once again this mechanism fails to adequately explain either the 7-10 minute lag before any growth, or the requirement for protein synthesis. A third model suggests that the effects of auxin are mediated by the constant traffic of vesicles from the endoplasmic reticulum and the golgi (Cleland, 1988). In this model the auxin activated ATPases are located on one of these organelles, and the effect of the auxin is to cause acidification of the lumen of the compartment. Each new vesicle formed will be carrying protons, while those formed before the effect of auxin will be neutral, thus acidification will occur when the former vesicles move to the cell membrane and release their contents. The requirement for protein synthesis can be explained by the need for new protein to be incorporated into the vesicles. Thus this theory explains all the effects of applied auxin; however, there is little direct evidence to support it, and so the primary mode of auxin action is still largely unknown.
1.1.2 Auxin Binding Proteins

All the above theories involve in one way or another an auxin binding protein. The first convincing evidence for an auxin binding protein appeared in 1972 (Hertel et al., 1972), but it was not until 1985 (Lobler and Klambt) that purification was achieved. The protein was a dimer with a molecular weight of 44 kDa and a binding affinity towards naphthalene-1-acetic acid of $5.7 \times 10^{-8}$M. Since then many other proteins able to bind auxin have been found (Hesse et al., 1989; Inohara et al., 1989; Tillmann et al., 1989; Prasad and Jones, 1991). In many cases the function of the binding protein remains completely unknown.

In one case an auxin binding protein has been isolated for which the activity is known (Campos et al., 1992). This is a protein from maize, which was found by photoaffinity labelling, that is distributed in both the cytosol and the microsomes. The protein is a $\beta$-glucosidase which shows high substrate specificity towards indoxy-O-glucosides. It was suggested that the enzyme may be involved in the hydrolysis of auxin conjugates.

Putative functions of the auxin binding proteins have been examined by comparing the effects of cell-free extracts treated with antibodies to the auxin-binding protein with cell-free extracts that were untreated (Andre and Scherer, 1991). This study illustrated the role of one auxin-binding protein in controlling the level of activation of phospholipase A. A similar study yielded preliminary evidence that GTP binding proteins were involved in auxin signal transduction (Zaina et al., 1990).

Thus, many possible auxin binding proteins are being found, yet most are of unknown function. Until the function and mode of action, especially with regards to interaction with auxin-mediated gene transcription, are understood, the actions of auxin will be at best only surmise.
1.2 Biosynthesis

1.2.1 Tryptophan as a Precursor to IAA

The close structural relationship of tryptophan to indoleacetic acid and its occurrence in all plants made tryptophan the obvious precursor to indoleacetic acid. The probability of this was strengthened by the discovery by Thimann (1935) that the fungus *Rhizopus suinus* formed indoleacetic acid when supplied with exogenous tryptophan. This was confirmed by radiotracer studies in which $^{14}$C-tryptophan was shown to form $^{14}$C-indoleacetic acid (Dannenburg and Liverman, 1957). Final proof was given by Erdmann and Schiewer (1971) by incubating plants with $^3$H-serine and $^{14}$C-indole or $^3$H,$^{14}$C-tryptophan. They showed that the ratio of $^3$H to $^{14}$C in extracted IAA was the same in both cases. Since tryptophan is formed by the condensation of serine and indole in the shikimate pathway, then the expected ratios would be the same; if the indoleacetic acid was formed from indole alone then the ratios would be different. The existence of other pathways not involving tryptophan was ruled out by a similar set of experiments using a tryptophan synthetase inhibitor and radiotracers (Heerkloss and Libbert, 1976).

L-Tryptophan levels in plants may be several orders of magnitude greater than indoleacetic acid levels (Kutacek, 1985; Schneider *et al.*, 1972). However it is not known what portion of the tryptophan is available to indoleacetic acid biosynthetic enzymes and it is possible that only a single small pool of tryptophan is involved in the indoleacetic acid synthesis. Pools of tryptophan are likely to exist in the chloroplast, mitochondria and cytoplasm, where it is required for
protein synthesis, and in the vacuole and lysosomes as a product of protein hydrolysis.

There is evidence that D-tryptophan may also be involved in indoleacetic acid synthesis, through its conversion from the L isomer. Law and Hamilton (1984) showed that treatment with Gibberellin A3 (GA3) induced greater stem elongation rates and increased, by up to tenfold, the amount of free indoleacetic acid in young leaves and internodes of dwarf pea seedlings. Law (1987) showed that stem segments excised from light grown dwarf pea plants elongated in the presence of D-tryptophan, but elongation in the presence of L-tryptophan also required GA3; he also showed that growth in the presence of L-tryptophan or of D-tryptophan with GA3 was inhibited by the D-amino acid aminotransferase inhibitor D-cycloserine, suggesting that blocking the transamination of D-tryptophan was preventing the formation of indoleacetic acid from L-tryptophan and D-tryptophan. Similar results have been obtained with barley (Tsurusaki et al., 1990). However, it may be possible that the D-amino acid aminotransferase activity was essential to plant growth in some other way.

The tryptophan racemase has been detected (Miura and Mills, 1971; Aldag and Young, 1970), but it has not been purified and the stimulating effects of GA3 have not been investigated. A D-tryptophan aminotransferase has also been recently purified from Alaska peas (McQueen-Mason and Hamilton, 1989). It was shown to be located in the plastid and had no activity with L-tryptophan and to be far more active in dark grown than light grown seedlings.

Recently, it has been suggested that tryptophan is not the precursor of indoleacetic acid (Wright et al., 1991; Baldi et al., 1991). This is based on the results from labelling indoleacetic acid and tryptophan by supplied labelled precursors to mutant plants without tryptophan
synthase. Labelling patterns showed that more label was found in indoleacetic acid than in tryptophan. However, the mutant species involved are both leaky mutants, and the possible effects of compartmentation of indoleacetic acid biosynthesis are not considered.

The conversion of tryptophan into indoleacetic acid involves modification of the sidechain which can occur through a number of different pathways. Which pathway is utilised in plants remains uncertain, and it is possible that different plants use different routes of synthesis, or that more than one pathway occurs in a single plant.

1.2.2 Indolepyruvate Pathway

\[ \text{Trp} \rightarrow \text{IPyA} \rightarrow \text{IAAld} \rightarrow \text{IAA} \] (see Fig. 1.3)

Indolepyruvate can be produced from tryptophan either by transamination or oxidative deamination. The identification of indolepyruvate in plants is difficult due to its instability and low concentration within the plant. Although provisionally identified by paper chromatography in plant pea seedlings as early as 1961 (Libbert and Brunn), it has only recently been unequivocally identified by mass spectrometry (Badenoch-Jones et al., 1984; Cooney and Nonhebel, 1989). Metabolic studies have also suggested the involvement of indolepyruvate in indoleacetic acid biosynthesis. \(^{14}\text{C}-\text{tryptophan applied to excised tomato shoots (Schneider et al., 1972)}\) and crude cell free extracts of barley (Wightman, 1973) resulted in the identification of radiolabelled indolepyruvate, indoleacetaldehyde and indoleacetic acid.

Enzymes for both transamination and oxidative deamination have been found. An aminotransferase which reacts with the aromatic L-
Fig. 1.3. Possible Routes of Indoleacetic Acid Biosynthesis.

Pathways for the synthesis of indoleacetic acid from tryptophan:

(A) Indolepyruvate pathway (Section 1.2.2)

(B) Tryptamine pathway (Section 1.2.3)

(C) Indoleacetaldoxime pathway (Section 1.2.4).
amino acids has been isolated from mung bean shoots (Wightman and Cohen, 1968; Truelson, 1972). Truelson (1973) further showed its existence in many plant species, including examples from at least 16 families. However, plant aminotransferases are not highly specific and many other aminotransferases are able to form indolepyruvate from tryptophan, including aspartate, alanine and ornithine aminotransferases (Wightman and Forest, 1978), making it possible that the aminotransferase is not involved in indoleacetic acid biosynthesis.

An L-tryptophan dehydrogenase, capable of the oxidative deamination of tryptophan to indolepyruvate, has been discovered recently in pea, maize, spinach and tomato seedlings (Kutacek, 1985). It is specific to L-tryptophan, even the closely related phenylalanine cannot serve as a substrate (Kutacek and Terziivanova-Dimova, 1991). It catalyses the reversible deamination of tryptophan and the amination of indolepyruvate in the presence of NAD or NADP; the latter reaction proceeds more favourably.

The next step in the pathway is the decarboxylation of indolepyruvate to form indoleacetaldehyde. This compound was demonstrated to have biological activity in several test systems by Larsen and Rajagopal (1964). It has been isolated in shoots of Pisum and Helianthus seedlings by comparison of chromatographic and UV spectra with authentic indoleacetaldehyde (Rajagopal, 1967). It has also been detected in tomato (Libbert et al., 1970) and pea (Brown et al., 1976) by gas chromatography. Its formation from indolepyruvate was suggested by metabolic studies using radiotracers (Gibson et al., 1972a).

The enzyme catalysing the reaction, indolepyruvate decarboxylase, has been reported to be present in tomato shoots (Gibson et al., 1972a), but no further purification nor kinetic studies have occurred; indolepyruvate decarboxylase activity has also been found in crude
preparations of barley (Schneider et al., 1972) and pea (Suzuki et al., 1981).

The indolepyruvate decarboxylase from the bacteria Enterobacter cloacae has been isolated and sequenced by molecular cloning (Koga et al., 1991). The gene showed considerable similarity to pyruvate decarboxylase in yeast, and when expressed in E. coli it was able to convert indolepyruvate to indoleacetaldehyde; and the existence of this gene was shown to be the only requirement for the formation of indoleacetic acid from tryptophan in both E. coli and E. cloacae.

1.2.3 Tryptamine Pathway

Trp → TNH₂ → IAAld → IAA (Fig. 1.3)

Tryptamine is formed by the decarboxylation of tryptophan; it has been found to occur naturally in tomato and barley seedlings, using paper chromatography and UV and infrared spectra (Schneider et al., 1972). The possible role of tryptamine in indoleacetic acid biosynthesis was suggested by its biological activity, following a lag period, in Avena coleoptiles (Schneider and Wightman, 1974). The conversion of ¹⁴C-tryptophan to labelled tryptamine, indoleacetaldehyde and indoleacetic acid was shown in tomato shoots (Gibson et al., 1972a) and a cell free system of tobacco (Phelps and Sequiera, 1968), as well as the conversion of ¹⁴C-tryptamine to labelled indoleacetic acid in cucumber seedlings (Sherwin and Purves, 1969).

A tryptophan decarboxylase has been isolated from cucumber (Sherwin, 1970) and tomato and barley (Gibson et al., 1972a,b). In the Gramineae, tryptophan decarboxylase is involved in the formation of
two important antineoplastic indole alkaloids (Songstad et al., 1990), and tryptophan decarboxylase from *C. roseus* has been purified to homogeneity (De Luca et al., 1989). It occurs as a homodimer with a requirement for pyridoxal phosphate, and is specific for tryptophan. The gene for tryptophan decarboxylase has been cloned and multiply expressed in tobacco (Songstad et al., 1990), resulting in a plant with 250 fold levels of tryptamine, but unchanged indoleacetic acid levels and growth and development were normal compared to wild type plants.

The conversion of tryptamine to indoleacetaldehyde is catalysed by plant amine oxidases. Percival and Purves (1974) found several forms of amine oxidase in cucumber seedlings, one of which showed a high activity with tryptamine, but they did not show its relevance to indoleacetic acid biosynthesis.

While tryptamine has been shown to be a possible precursor to indoleacetic acid (Sherwin and Purves, 1969), Magnus and co-workers (1973) found that, compared with incubations with indoleacetaldehyde and indoleethanol, tryptamine formed only a small trace of indoleacetic acid in pea, while large amounts were converted to indoleethanol and indoleethanol conjugates.

**1.2.4 Indoleacetaldoxamine Pathway**

\[
\begin{align*}
\text{Trp} & \rightarrow \text{IAOx} \\
\text{IAA} & \rightarrow \text{IAN} \\
\text{IAAld} & \rightarrow \text{IAA} (\text{Fig. 1.3})
\end{align*}
\]

Indoleacetaldoxime has been identified as a natural constituent of cabbage by chromatography and UV spectra (Kindl, 1968) and by mass
spectrometry (Conn, 1981). Labelled indoleacetaldoxime was formed from exogenous $^{14}$C-tryptophan, and has been shown to promote growth in wheat and pea tissue (Fawcett, 1964).

Indoleacetaldoxime can be converted to indoleacetaldehyde in oats (Rajagopal and Larsen, 1972) and in tomato (Schneider and Wightman, 1974) or indoleacetonitrile in cabbage (Kutacek and Kefeli, 1970) and in wheat and corn (Wightman, 1962). Indoleacetonitrile promotes growth in some plants: oat (Bentley and Houseley, 1962), wheat, maize (Seeley et al., 1956), cabbage, radish and turnip (Ballin, 1962). However several species, including pea (Seeley et al., 1956), broad bean (Thimann, 1955) and tomato (Wightman, 1962) are not affected by indoleacetonitrile, apparently because indoleacetic acid cannot be formed from indoleacetonitrile. It is of interest that tomato leaves do not contain detectable indoleacetaldoxime (Cooney and Nonhebel, 1989).

The enzyme which converts tryptophan to indoleacetaldoxime has been found as a plasma membrane bound protein in Chinese cabbage, maize, sunflower, tobacco and pea (Ludwig-Miller and Hilgenburg, 1988). The enzyme which converts indoleacetaldoxime to indoleacetaldehyde has been detected in Chinese cabbage (Helmlinger et al., 1987) and the enzyme which converts indoleacetaldoxime to indoleacetonitrile was detected (Mahadevan, 1963) in banana leaves. This enzyme has only been found in a limited number of families and is not highly substrate specific (Mahadevan and Thimann, 1964; Morgan et al., 1966).

Glucobrassicin is an indoleglucosinolate found only in the *Brassicaceae* and related families. It is also formed from indoleacetaldoxime (Kutacek and Kefeli, 1968). The biosynthetic route of synthesis from tryptophan has been studied by radiolabelling and glucobrassicin can be converted to indoleacetonitrile and may thus be a special pathway for indoleacetic

### 1.2.5 Indoleacetaldehyde Conversion

The final step of most of the pathways mentioned above is the conversion of indoleacetaldehyde to indoleacetic acid. This has been demonstrated by radiotracer studies in both whole plants and callus tissue culture explants (Rajagopal, 1967; Wightman and Cohen, 1968; Libbert *et al.*, 1970; Gibson *et al.*, 1972a). Two different enzymes have been reported which could carry out the reduction.

The first is a NAD-dependent indoleacetaldehyde dehydrogenase found in cytoplasmic preparations from mung bean seedlings (Wightman and Cohen, 1968) and in tomato shoots (Wightman, 1973). The second is a molecular oxygen-requiring indoleacetaldehyde oxidase found in *Avena*, pea and cucumber seedlings (Bower *et al.*, 1976; Rajagopal, 1971), which was shown by inhibition studies to be strongly specific to indoleacetaldehyde.

As well as conversion to indoleacetic acid, indoleacetaldehyde can be reversibly transformed to indoleethanol. Indoleethanol was first detected in cucumber, using mass spectrometry (Rayle and Purves, 1967), it has subsequently been identified in pea seedlings (Brown *et al.*, 1986, tomato shoots (Schneider *et al.*, 1972), sunflower seedlings (Rajagopal, 1967) and pine (Sandburg, 1984). Radiolabelled indoleethanol was detected in *Phycomyces* after exogenous $^{14}$C-tryptophan was applied (Schramm *et al.*, 1987). Applied indoleethanol does show auxin activity, but it is thought that this is due to the conversion to indoleacetaldehyde and formation of indoleacetic acid.
(Magnus et al., 1973). Most indoleethanol is localised in the chloroplast (Brown et al., 1986) and it exists both in free form and also as conjugates such as O-acetyl indoleethanol and indoleethanol-β-D-glucopyranoside (Lacan et al., 1985).

Several distinct enzymes have been found which catalyse the formation of indoleethanol. A cytosolic NADH-dependent indoleacetaldehyde reductase was detected in cucumber seedlings (Bower et al., 1976), a microsomal NADPH-dependent indoleacetaldehyde reductase was purified from Cucumis sativus (Brown and Purves, 1976) and a cytosolic NADPH-dependent enzyme also from Cucumis sativus (Ludwig-Miller and Hilgenburg, 1989), but no chloroplast compartmented enzyme has been reported.

The reconversion of indoleethanol to indoleacetaldehyde occurs rapidly and an enzyme catalysing the reaction has been partially purified from Cucumis sativus and characterised (Vickery and Purves, 1972; Percival et al., 1973). It is an alcohol dehydrogenase, which is not NAD dependent and possibly is a flavoprotein with full activity dependent on metal ion and sulfhydryl groups. It is inhibited by both indoleacetaldehyde and indoleacetic acid.

It has been suggested that indoleethanol may act as a storage pool for indoleacetic acid, rather than as a separate pathway for biosynthesis (Schramm et al., 1987; Brown and Purves, 1980). This is supported by the fact that the indoleethanol oxidase in Cucumis sativus is subject to feedback inhibition (Percival et al., 1973), and also an excess of exogenous 14C-tryptophan applied to tomato tissue led to a small increase in the pool size of free indoleacetic acid, but increased indoleethanol levels 2 to 3-fold (Gibson et al., 1972a). Hence it is possible that indoleethanol may have a role in the regulation of indoleacetic acid levels.
1.2.6 Which Pathway?

There are at least three pathways from tryptophan to indoleacetic acid that might occur in higher plants: the indolepyruvate, tryptamine and indoleacetaldoxime pathways. This immediately raises the question of how indoleacetic acid is synthesized. One of the pathways may predominate or some combination of the three may be involved. There are a number of problems with the experiments to date which make answering this question difficult.

Firstly, unequivocal identification of indoleacetic acid and its precursors. The best method for identification of such small organic molecules is by mass spectometry (Morgan and Durham, 1983), usually performed in conjunction with gas chromatography. However, most reports identifying indoleacetic acid and its precursors use paper chromatography combined with chemical or biological assays, methods which do not explicitly identify the compounds.

Secondly, there is the problem of a large number of enzymes with low specificities which can carry out the reactions necessary to form indoleacetic acid. This multispecificity raises the question as to the principle function of the enzyme in vivo. It is further complicated by the fact that many of the intermediates are so unstable that they break down non-enzymically to indoleacetic acid, for example indolepyruvate breaks down rapidly to form a number of products including indoleacetic acid (Bentley et al., 1956). Also to be considered is the ability of bacteria to form indoleacetic acid from tryptophan: metabolic studies using nonsterile plant tissue and radiolabelled tracer may result in tracer conversion to indoleacetic acid by bacteria (Libbert et al., 1966, 1968).
Finally, there are problems with equilibration and compartmentation of enzymes and radiolabelled tracers. In crude homogenates, enzymes which are normally separated by organelle membranes can be found together, thus forming pathways that do not exist \textit{in vivo}. Similarly, \textit{in vivo} tracer experiments are limited by the ability of the tracer to be transported to, and equilibrate with, all the endogenous pools of the compound, especially those at the site of indoleacetic acid biosynthesis; the tracer may be metabolised by nonspecific enzymes before reaching the site of synthesis.

Even with these problems, a single pathway to indoleacetic acid biosynthesis might be postulated: the indolepyruvate pathway. The indoleacetaldoxime pathway is unlikely, but still possible, since indoleacetaldoxime has not been detected in some species (Wightman, 1962). The tryptamine pathway is unlikely because large amounts of tryptamine formed in the transgenic tobacco with extra copies of the tryptophan decarboxylase gene did not produce more indoleacetic acid than wild type plants (Songstad \textit{et al.}, 1990).

There is evidence in favour of the indolepyruvate pathway (Cooney and Nonhebel, 1991a,b). Tomato seedling shoots were equilibrated in $^{2}\text{H}_2\text{O}$ and then tryptophan, indolepyruvate and indoleacetic acid were extracted and quantified using gas chromatography-mass spectrometry. Since the $^{2}\text{H}_2\text{O}$ is such a small molecule it readily equilibrates across membranes and into all compartments. The labelling patterns and amount of labelling of the three compounds as they incorporated the deuterium were compared over a period of time. The labelling patterns of indolepyruvate and indolepyruvate were sufficiently related to support the hypothesis that indoleacetic acid was synthesized from indolepyruvate. Hence this pathway is the most likely to occur \textit{in vivo}. 

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1.3 Regulation of IAA Levels

1.3.1 Is Control of IAA Levels Important?

The concept of hormonal control is based on the idea that the concentration of a hormone or growth factor at the cell responding to the substance determines the magnitude of the response. However, a physiological concentration related response to plant growth regulators has not been established. In bioassays growth regulators yield a response over four to five orders of magnitude of concentration, but observed changes of endogenous growth regulator concentration are only one order of magnitude.

As an alternative to the concentration dependent response, Trewavas (1981, 1982, 1991) suggested that the response to a plant growth regulator could depend on sensitivity: the competence of a tissue to respond to the growth regulator. Thus the response of the plant may be more dependent on the presence or absence of a receptor (or changes in its binding affinity) than on the concentration of the growth regulator. Variation in sensitivity may occur during the development of plant tissues, thus causing changes in the effects of the growth regulator. An example of this is the precocious ripening of fruit using exogenous ethylene. Very immature fruit can be made to ripen using high levels of ethylene, but as normal development proceeds the amount of ethylene required to elicit a saturation response diminishes (McGlasson et al., 1978).

Firn (1986) pointed out that a change of sensitivity could result from a number of different processes or a combination of them. As well as changes in the number of receptors and in their binding affinities,
factors which might be involved in changing sensitivity might include: ability for the tissue to take up the growth factor, overall capacity of the tissue to respond to occupied receptors and the amounts and activities of growth receptor metabolising enzymes. Thus the anomalous results of bioassays may be caused by limited uptake or metabolism of the growth factor.

The fact that concentration does play a role in controlling a plant's response to a plant growth regulator is illustrated by several examples. The first of these is the cause and effects of the plant disease crown gall. This is induced by the bacterium Agrobacterium tumefaciens, which transfers into the plant a plasmid, the Ti plasmid, which is integrated into the plant nuclear genome (Mayerhofer et al., 1991). Included amongst the genes on the plasmid are several which encode enzymes for indoleacetic acid and cytokinin biosynthesis, using pathways not usually occurring in plants (Schroder et al., 1984). This results in greatly increased levels of indoleacetic acid and cytokinin and manifests itself as tumorous growths on the plant. Thomashaw and co-workers (1986) have demonstrated that the overproduction of growth regulators caused the development of the tumours.

A second example is the comparison of indoleacetic acid levels in various phenotypes of pea seedlings (Law and Davies, 1990). The phenotypes investigated were the wild type, dwarf and extreme dwarf and several slender varieties and a direct relationship was shown between the height above the second node and the indoleacetic acid content of the stem elongation zone, implying a direct correlation between concentration of indoleacetic acid and seedling growth.

A third example is the direct measurement of indoleacetic acid levels in Zea mays seedlings in comparison to growth (Bandurski et al., 1990). In these experiments the amount of indoleacetic acid was
decreased by decapitation of the seedlings, and the concentration of indoleacetic acid, determined by gas chromatography-mass spectroscopy, was compared with the growth of the seedlings. The resulting Michaelis-Menten plot was linear, and significantly when indoleacetic acid concentration and growth were decreased by exposure to red light, the result fell on the same line in the Michaelis-Menten plot. The conclusion is that the primary control of growth is by the amount of indoleacetic acid (Bandurski et al., 1990).

Thus, these example show that there is a dependence between the concentration of auxin and its effects on growth; while sensitivity may play some role in the magnitude of the response, auxin levels are still important.

1.3.2 Examples of Control of Plant Growth Regulators

Ethylene Biosynthesis

Ethylene is synthesized from methionine as shown in Fig. 1.4. The 5'-methylthioadenosine formed as a byproduct is recycled through a number of steps back to methionine and adenosine. As part of the normal life of a plant ethylene production is induced during certain stages of growth including germination, ripening of fruits and senescence of flowers. Production can also be induced by external factors such as wounding and chemicals including auxin (Yang and Hoffman, 1984).

Climacteric fruits are characterised by a surge of ethylene production at the onset of ripening, and this ethylene pays an essential role in the ripening (Abeles, 1973). It was shown that ACC levels were
Fig. 1.4. Biosynthesis of Ethylene.

The biosynthesis of ethylene, consisting of two sections: the methionine cycle, in which methionine and ATP are combined to form ACC, the methionine and ATP are reformed. Ethylene is formed from ACC in a single reaction step.
ATP

Methionine

S-adenosyl-L-methionine

S-adenosylmethionine synthase

5'-methylthioadenosine

Ethylene Forming Enzyme (EFE)

1/2 O₂

CO₂ + HCN

Ethylene
very low in unripe fruit, but at the onset of ripening these levels increased hugely (Apelbaum et al., 1981). Kende and Boller (1981) then showed that the activity of ACC synthase was not detectable before ripening, but markedly increased at ripening. However application of ACC to unripe fruit exhibited only a small increase in ethylene (Yang, 1981), implying that not only is the ACC synthase a limiting enzyme, but that the ETE may also be controlled to a small extent.

The fact that auxin promotes ethylene production was discovered in 1935 (Zimmerman and Wilcoxon); in vegetative tissues the rate of ethylene production is thought to be regulated by the free auxin level: higher rates of ethylene synthesis occur in tissues with more auxin (Abeles, 1973). It was shown that the effect of auxin was in regulating the conversion of SAM to ACC (Yu and Yang, 1979), which was the rate limiting step of the pathway. The activity of the ACC synthase was shown to increase markedly in line with auxin induced ethylene synthesis (Yoshii and Imaseki, 1981) and that this increase could be inhibited by cycloheximide, actinomycin D and α-amanitin (Yoshii and Imaseki, 1982), implying that auxin increases ethylene synthesis through RNA and protein synthesis. For comprehensive control of ethylene synthesis, ACC synthase turn over would have to be high to allow for rapid decreases in the rate of synthesis; this has been shown to be true, the enzyme has a half life of about 25 minutes (Kende and Boller, 1981; Yoshii and Imaseki, 1982).

Ethylene has also been shown to be able to regulate its own biosynthesis. Liu and coworkers (1985) have shown that exposing the unripe climacteric fruit to ethylene does not increase ethylene synthesis, but does increase the ability of the tissue to convert ACC to ethylene. Autoinhibition has also been shown to occur: in wound induced ethylene synthesis of the flavedo tissue of citrus fruits, ethylene synthesis and
ACC levels increase markedly normally, but in the presence of exogenous ethylene there is little change in both; upon the addition of exogenous ACC normal ethylene production occurred (Riov and Yang, 1982). This decrease in ACC production was not paralleled by a decrease in ACC synthase levels, suggesting that it was due to inhibition of ACC synthase activity (Riov and Yang, 1982).

The control of the amounts of ACC synthase by regulation of the mRNA levels of the enzyme has lead to studies on the gene for the enzyme. In both zucchini (Huang et al., 1991) and tomato (Olson et al., 1991) multiple copies of the gene have been discovered, each of which is induced by different factors.

Thus, the major element in regulation of ethylene biosynthesis is the control of ACC synthase levels by control of RNA synthesis and a high turnover rate. This results in dramatic changes in ethylene production, finer control is carried out by inhibition of the ACC synthase and by control of the EFE activity (Yang and Hoffman, 1984).

**Gibberellin Biosynthesis**

The biosynthesis of gibberellins can divided into three stages: the synthesis of ent-kaurene, the conversion of ent-kaurene to GA$_{12}$-aldehyde (Fig.1.5) and the biosynthesis after GA$_{12}$-aldehyde. All three of these stages seem to have at least one kind of control mechanism, to regulate the levels of gibberellin.

The synthesis of ent-kaurene is a two step process, starting with the isoprenoid geranylgeranyl pyrophosphate, which is catalyzed by two separable enzymes known as ent-kaurene synthase A and B (Fig. 1.5). However, while the enzymes are separable there is evidence that the
The first two stages of gibberellin biosynthesis. The first stage is formation of ent-kaurene from geranylgeranyl pyrophosphate, the steps A and B are catalysed by the A- and B-enzymes of ent-kaurene synthetase.

The main steps of the second stage of biosynthesis, the formation of gibberellin A_{12}-aldehyde, including the major branchpoints are also shown.
Geranylgeranyl pyrophosphate

Copalyl pyrophosphate

ent-kaurene

Oxidation products

GA_{12}-aldehyde
two enzymes act in concert: when $[^{14}\text{C}]$GGPP and $[^{3}\text{H}]$CPP were used simultaneously as substrates for the AB enzyme, the $^{14}\text{C} : ^{3}\text{H}$ ratio in the ent-kaurene formed was 10-13 times greater than would be expected if the $[^{14}\text{C}]$CPP formed had equilibrated with the exogenous pool (Duncan and West, 1981).

There is evidence that the activity of ent-kaurene synthase varies with development of the plant. In cell free extracts of Pharbitis nil seeds, the ent-kaurene synthase activity is the greatest during the period of seed development in which the gibberellin content is the greatest (Barendse et al., 1983). It has also been shown that in pea seedling there is a strong correlation between the growth potential of a tissue and the level of ent-kaurene synthase in that tissue (Chung and Coolbaugh, 1986).

The next stage in gibberellin biosynthesis, the conversion to $\text{GA}_{12}$-aldehyde is carried out in one of the microsomal compartments, probably the endoplasmic reticulum (Graebe, 1980), separate from the cytoplasmic or chloroplast location of ent-kaurene synthase. In addition to this compartmentation, the pathway (Fig 1.5) also has two side branches, which are irreversible (Graebe, 1987) leading to removal of unwanted precursors to gibberellins.

The biosynthesis of gibberellins after $\text{GA}_{12}$-aldehyde is carried out by a number of low specificity cytosolic enzymes (Hedden and Graebe, 1982), responsible for two sets of reactions. The first set of reactions is the conversion of the aldehyde to the bioactive $2\beta$-hydroxy $\text{C}_{19}$ gibberellin, as shown in Figure 1.6. The second set of reactions, which occur simultaneously with the first, is the addition of extra hydroxyl groups at one or both of $\text{C}_3$ and $\text{C}_{13}$. Despite the low specificity of the enzymes involved in these transformations, there is evidence for control of gibberellin biosynthesis at this stage.
Fig. 1.6. Final Steps of Gibberellin Biosynthesis.

The conversions involved in forming bioactive gibberellins (the C\textsubscript{19} GAs) from gibberellin A\textsubscript{12}-aldehyde. Initially R1 and R2 are both hydrogens, but the two groups may be replaced by hydroxyl groups by nonspecific enzymes at any stage during the pathway.
Spinach is a long day rosette plant, with the stem growth occurring in the change from short day to long day mediated by gibberellins (Zeevaart, 1981). The conversion of GA$_{12}$-aldehyde to the bioactive GA$_{29}$ has been found to occur by the pathway:

$$\text{GA}_{12} \rightarrow \text{GA}_{53} \rightarrow \text{GA}_{44} \rightarrow \text{GA}_{19} \rightarrow \text{GA}_{20} \rightarrow \text{GA}_{29}$$  (Gilmour et al., 1986).

The difference in the levels of the gibberellins between short day and long day spinach was found to be a much higher level of GA$_{19}$ in short day plants, while GA$_{20}$ and GA$_{29}$ levels were increased in long day plants (Metzger and Zeevaart, 1980). Gilmour and coworkers (1986) showed that this change in the relative amounts of the various gibberellins was due to an increase in the activity of the enzymes converting GA$_{53}$ and GA$_{19}$ in long day condition; and the change in enzyme activity was proved not to be due to the effects of enzyme activators or inhibitors, suggesting a change in enzyme levels.

Hence, gibberellin biosynthesis seems to be controlled by many mechanisms operating at many levels at different periods. There is compartmentation of the entire process, metabolic channelling of ent-kaurene synthesis and the control of enzyme activities in concert with plant or plant tissue development.

1.3.3 Mechanisms For Control of Indoleacetic Acid

With indoleacetic acid there are several ways in which the concentration of indoleacetic acid could be controlled. These are through conjugation of the growth regulator, by control of its metabolism or by control of its synthesis.

Conjugation of indoleacetic acid was first demonstrated in 1935 (Cholodny), with the percentage of conjugation varying from 5% in
Avena species to 90% in Zea species (Cohen and Bandurski, 1982). The conjugates have a number of possible in vivo roles, including transport of indoleacetic acid in an inactive form (Komoszynski and Bandurski, 1986; Chisnell and Bandurski, 1988) or acting as a storage pool for indoleacetic acid, providing a way of saving indoleacetic acid in an inert form until it is required, thus allowing rapid release of the plant growth regulator. Hangarter and Good (1981) demonstrated that indoleacetic acid amide conjugates are metabolised to free indoleacetic acid and that their biological activities are related to the rate of conjugate hydrolysis. Bialek and co-workers (1983) showed that when $^{14}$C labelled conjugates were applied to the base of bean stems the resulting indoleacetic acid bioassay response was proportional to the free $^{14}$C-indoleacetic acid recovered from the tissue.

It is also been suggested that the conjugates are involved in the homeostasis of indoleacetic acid (Cohen and Bandurski, 1982), with the reversible synthesis and hydrolysis of indoleacetic acid forming an homeostatic mechanism for regulating indoleacetic acid concentrations. Enzymes capable of both synthesis and hydrolysis have been isolated (Hall and Bandurski, 1986; Michalczuk and Bandurski, 1982; Leznicki and Bandurski, 1988). It has also been that when photoinhibition was used to decrease the growth rate of Zea mays by 30% to 40%, a decrease of free indoleacetic acid and an concomitant increase of conjugates was observed (Bandurski et al., 1977).

The metabolism, or oxidation, of indoleacetic acid, like its biosynthesis, is not totally understood. There are two possible types of pathways that have been detected: the decarboxylation pathway and the non-decarboxylation or oxindoleaceticacid/dioxindoleacetic acid pathway.
Fig. 1.7. Decarboxylative Pathway of Indoleacetic Acid Catabolism.

The two main pathways and products of the oxidative decarboxylation pathway of indoleacetic acid catabolism by plant peroxidases.
3-hydroxymethyl-oxindole

indole-3-methanol

3-methyleneoxindole

indole-3-aldehyde

indole-3-carboxylic acid
The decarboxylation pathway (Fig. 1.7) involves modification of the acetic acid group: initially decarboxylation and then oxidation. It is catalysed by peroxidases from many species, and often each plant species has multiple peroxidase isoenzymes (Sembdner et al., 1981). These peroxidases are not very specific and the ratio of the final products depends on enzyme/substrate ratio, pH and cofactors, including Mn$^{2+}$ and 2,4-dichlorophenol (Reinecke and Bandurski, 1981).

The nondecarboxylation pathway (Fig. 1.8) involves modification of the indole ring, leaving the acetic acid side chain untouched. This pathway has only been shown to occur in two species: broad bean (Tsurumi and Wada, 1980), corn (Reinecke and Bandurski, 1983; Nonhebel and Bandurski, 1984) although both oxindole and dioxindole have been found in rice, suggesting the pathway is operative in rice also (Kinashi et al., 1976). However, measurements of $^{14}$CO$_2$ evolution from plant fed with 1-$^{14}$C-indoleacetic acid showed that only 40% of the indoleacetic acid broke down to give off carbon dioxide (BeMiller and Colilla, 1972), suggesting that the decarboxylative pathway is not the sole pathway of indoleacetic acid catabolism and a non-decarboxylative pathway must occur.

In the case of young Zea mays seedlings the biosynthesis, conjugation and metabolism of indoleacetic acid has been thoroughly studied (Epstein et al., 1980; Nowacki and Bandurski, 1980; Pengelley and Bandurski, 1983; Reinecke and Bandurski, 1983). The pool sizes and transport of indoleacetic acid, tryptophan, the indoleacetic acid conjugates and possible oxidation products were measured and the rate of metabolic turnover calculated. From these studies it was possible to show that the transport and subsequent hydrolysis of seed conjugates was sufficient to meet the indoleacetic acid requirements of the growing seedling (Reinecke and Bandurski, 1987).
Fig. 1.8 Nondecarboxylative Pathway of Indoleacetic Acid Catabolism.

The three different pathways of nondecarboxylative indoleacetic acid degradation found to date, and the plant they were observed in:

1. *Zea mays* (Reinecke and Bandurski, 1983)
2. *Vicia faba* (Tsurumi and Wada, 1980)
oxindole-3-acetic acid

R=H=oxindole-3-acetic acid
R=OH=dioxindole-3-acetic acid

7-hydroxyoxindole-3-acetic acid glucoside

R=H=5-hydroxyoxindole-3-acetic acid
R=OH=5-hydroxydioxindole-3-acetic acid
However, the large pool of seed conjugates that are available to the very young Zea mays seedling does not exist in older plants, and the control of the indoleacetic acid levels may be quite different. As mentioned earlier the disease crown gall is caused by synthesis of excessive levels of indoleacetic acid and cytokinin. If either conjugation or metabolism of indoleacetic acid were the method of controlling its endogenous concentration, then it would be expected that the excess indoleacetic acid would be removed by conjugation or oxidation. Since this does not occur, then either these methods are not used to control indoleacetic acid levels, or the amounts produced by the crown gall are so great that the plant is unable to completely control the concentration. The levels of conjugated indoleacetic acid have been determined in some crown galls (Rausch et al., 1986) and are only marginally higher than those in normal tissue, suggesting that conjugates are not involved in controlling indoleacetic acid levels in normal plants.

Thus, it is likely that the control of indoleacetic acid levels is carried out by regulation of its biosynthesis. Understanding of how the synthesis of indoleacetic acid is regulated is hampered by lack of experimental results. The uncertainty of which pathway is involved in the synthesis, combined with the many isoenzymes capable of catalysing many of the postulated steps as well as the frequent low specificity of those enzymes makes understanding of the control of indoleacetic acid biosynthesis very difficult.

To be certain whether an enzyme does play a role in the synthesis of indoleacetic acid, it is necessary to correlate the activity of the enzyme with the concentrations of the product of the enzyme and the indoleacetic acid. Since this has never been carried out with any of the putative enzymes or intermediates of indoleacetic acid, the ignorance of the control of indoleacetic acid synthesis, or even its correct pathway, is understandable.
1.3.4 General Metabolic Control as Related to Indoleacetic Acid

The simplest method of controlling the synthesis of a product of a metabolic pathway is to control the rate of reaction of the slowest step. In effect this is controlling the rate of one of the enzymes of the pathway. This is usually implemented by one or more of three mechanisms: covalent modification, regulatory proteins and feedback inhibition.

Covalent modification is the addition of chemical groups to activate or inhibit the enzyme as in phosphorylation of many growth factor receptors; or the cleavage of part of the enzyme to activate or deactivate it, as in the cleavage of trypsinogen to yield the active digestive enzyme trypsin.

Regulatory proteins can either stimulate or inhibit enzyme activity; an example of this is calmodulin, which acts as a calcium sensor, binding \( \text{Ca}^{2+} \) which leads to a structure modification to a form that binds to various enzymes, altering their activity. Covalent modification and regulatory proteins provide rapid large scale changes in rates of metabolic pathways with the use of very small triggering signals.

Feedback inhibition, in contrast, is a much simpler mechanism, whereby high levels of the final product of the pathway inhibit the activity of one of the enzymes. The usual enzyme that is inhibited is the first committed enzyme of the pathway, since this prevents the build up of other intermediates of the synthesis. An example of feedback inhibition is the biosynthesis of isoleucine. Isoleucine is formed from threonine, and the first enzyme involved, threonine dehydrogenase, is inhibited by isoleucine at a site distinct from the active site.
In indoleacetic acid biosynthesis, the putative first committed step is the conversion of tryptophan to indolepyruvate. This and the fact that all the intermediates beyond tryptophan are highly labile and able to breakdown non-enzymatically to indoleacetic acid makes this step the only likely step for feedback inhibition.

An alternative, but frequently effected, method of controlling the rate of one enzymic step is to change the levels of the enzyme. This is usually accomplished by altering the rate of enzyme degradation and the amount of mRNA available for transcription. An example of this is the regulation of ethylene by controlling levels of ACC synthase; the amount of ACC synthase is controlled by changing rates of transcription of mRNA in response to various external stimuli. As with feedback inhibition, the most likely point for this to occur is at the first committed step which, in indoleacetic acid biosynthesis, is the formation of indolepyruvate.

Alternative methods of controlling the rate of an enzymic pathway involve controlling the interactions between the various enzymes of the pathway. There are three major ways that this is realised: compartmentation, metabolic channelling and substrate cycles. Compartmentation is the physical separation of enzymes within differing organelles or portions of organelles within the cell. The selective permeability of membranes controls the passage of intermediates across the membrane, with specific carriers mediating transport into and out of the compartment.

Metabolic channelling results from association of enzymes, whether as a multifunctional protein with several different catalytic sites, or the existence of weak specific protein-protein interactions. This proximity allows more efficient conversion of the initial precursor to the product, because the intermediates do not have to diffuse as far to the next
catalytic site in the pathway. An example of this channelling is ent-kaurene synthetase, which catalyses the two step synthesis of ent-kaurene from geranylgeranyl pyrophosphate, as described in Section 1.3.2.

Substrate cycling is where two enzymes exist for the interconversion of two of the compounds on a metabolic pathway. These are sometimes known as futile cycles, since they allow recycling of the substrates, but can consume ATP or reducing equivalents. Control of the levels of the two enzymes involved allows for control of the levels of the two substrates and hence the amount of the final product. This occurs in carbohydrate metabolism with the conversion between fructose-6-phosphate and fructose-1,6-bisphosphate. The two enzymes involved, phosphofructokinase and fructose bisphosphatase, respond in opposite ways to the same allosteric effectors so that conditions activating one inhibit the other, and so the rate and direction of the metabolism is controlled.

All of these methods may play a role in control of the biosynthesis of indoleacetic acid. Tryptophan is synthesized in both the cytosol and the chloroplast, and pools are required in the chloroplast, the mitochondria and the cytosol for protein synthesis. Similarly pools exist where protein degradation occurs: the vacuole and lysosomes. Localisation of indoleacetic acid synthesis within the cell has not been determined, so there are many potential subcellular compartments. Since all the intermediates in the biosynthesis are so labile, some kind of compartmentation would be expected, and the potential for metabolic processing is great.

The possibility of substrate cycles can be pinpointed to two portions of the postulated biosynthesis pathway. The first of these is the cycling between indoleacetaldehyde and indoleethanol, with the later acting as
a storage compound as mentioned in Section 1.2.5. The second site is the conversion of tryptophan to indolepyruvate. Two reversible enzymes have been detected that are able to carry out this reaction, and an understanding of the control of these enzymes would greatly increase understanding of the control of indoleacetic acid biosynthesis.
1.4 Aminotransferases

Aminotransferases have been implicated in the biosynthesis of IAA as the enzyme catalysing the formation of indolepyruvate. Aminotransferases (also known as transaminases) are enzymes which catalyse the transfer of an amino group plus a proton and an electron pair from an amino donor compound to the carbonyl position of an amino acceptor compound. Aminotransferase activity was first observed in pigeon muscle tissue in 1937 (Braunstein and Kritzmann) and soon after was observed in plant tissue (Virtanen and Lane, 1938). The presence of aminotransferases has been found to be widespread and the range of amino acids used is great, with only proline and cysteine not capable of acting as a substrate for transamination among the free 'protein' amino acids (Forest and Wightman, 1972a).

1.4.1 Functions

The most obvious, and probably most important function of aminotransferases is in the synthesis of protein amino acids. Nitrogen is initially assimilated into glutamine and glutamate, and it can then be distributed to other compounds using aminotransferases. Kirk and Leech (1972) showed that isolated chloroplasts were able to synthesize nearly all amino acids when supplied with suitable amino group donor molecules.

The biosynthetic routes for most amino acids have been determined, and in many the final step is transamination of the 2-oxo
acid analogue of the amino acid. This is especially important with the formation of aspartate from oxaloacetate (Reed and Hess, 1975) and alanine from pyruvate (Bryan, 1976), with both enzymes involved in the regeneration of oxo acids as well as amino acid biosynthesis. Other amino acids synthesized from oxo acids are glycine from glyoxylate, valine from oxoisovalerate, leucine from oxoisocaproate and isoleucine from oxo-3-methylvalerate.

An aminotransferase is also involved in adding the amino group in the synthesis of other amino acids, although not as the final step of synthesis. Phosphoserine aminotransferase and serine aminotransferase are both responsible for the incorporation of nitrogen into serine (Bruin et al., 1970; Miflin et al., 1966). Histidine synthesis also involves an aminotransferase for the transamination of the amino group from glutamate (Miflin, 1980). Tyrosine and phenylalanine are both synthesized from prephenate in a two step route that involves an aminotransferase as either the first or the second step (Rubin and Jensen, 1979).

In an analogous manner aminotransferases are involved in forming non-protein amino acids, such as δ-aminolevulinic acid, a precursor in chlorophyll biosynthesis (Weinstein and Castelfranco, 1978), and many other nitrogen containing metabolites. These metabolites include alkaloids such as γ-coniceine (Roberts, 1977, 1978) and rosmarinic acid (De-Eknamkul and Ellis, 1987).

In addition to their direct roles in biosynthesis, aminotransferases also participate in various shuttle mechanisms as described below. Aminotransferases have also been implicated in the catabolism of many amino acids (Mazelis, 1980); indeed the catabolism of aspartate and glutamate is merely the reverse of their synthesis and is probably a
method of regenerating the oxo group donor molecules, oxaloacetate and 2-oxoglutarate.

Hydrogen transfer, or the transport of reducing equivalents, is required between the various subcellular compartments in plant cells due to the impermeable nature of organelle membranes to pyridine nucleotides (i.e. NADH and NADPH). This might be accomplished by a malate/oxaloacetate shuttle, however, other considerations suggest that the transamination of free amino acids, using a dicarboxylate transporter (Fig. 1.9) is the preferred route (Heber, 1974). Appropriate translocators and aminotransferase enzymes have been discovered for such a transport system in the chloroplast (Heber and Heldt, 1981). A similar scheme has also been postulated for the peroxisome (Rehfeld and Tolbert, 1972).

However, the major role of the chloroplast dicarboxylate transporter as an exporter of reducing units has been questioned. Using a dicarboxylate transporter deficient mutant, Somerville and Ogren (1983) demonstrated that its major function was the transfer of 2-oxoglutarate and glutamate across the chloroplast membrane for transamination in conjunction with photorespiratory nitrogen metabolism.

Photorespiration is the pathway, in C3 plants resulting in the wasteful oxidation of recent products of photosynthesis, due to the inherent oxygenase activity of ribulose bisphosphate carboxylase. The pathway (Fig. 1.10) occurs through three organelles. In the chloroplast, ribulose bisphosphate is oxidised to glycolate. This is taken to the peroxisome where it is oxidised to glyoxylate. The glyoxylate is transaminated to glycine, using both serine and glutamate as amine donors; the hydroxypyruvate formed from serine is reduced to glycerate, which reenters the Calvin cycle. The serine itself is formed in
Fig. 1.9. Dicarboxylic Acid Transport System.

The chemical transformations and intercompartmental movement of compounds involved in the dicarboxylic acid transport system, allowing the transport of reducing equivalents, formed in the chloroplast, into the cytosol.
The chemical transformations, and their compartmental locations, of photorespiration with the relationship to the dicarboxylic acid transport system.
the mitochondria from two molecules of glycine, with the release of carbon dioxide and ammonia. The carbon dioxide also reenters the Calvin cycle, while the ammonia is reassimilated into glutamine via glutamine synthetase.

In low glutamate conditions, the glyoxylate degrades nonenzymatically, producing high levels of toxic ammonia and carbon dioxide which prevents carbon fixation. Thus aminotransferases are involved in two stages of photorespiration. A serine:glyoxylate aminotransferase for removing glyoxylate and aspartate aminotransferases for the production of glutamate.

In C₄ plants, the photorespiratory pathway is avoided by the use of a carbon shuttle (Fig. 1.11). Atmospheric carbon dioxide is fixed by phosphoenol pyruvate into the C₄ dicarboxylic acid oxaloacetate. This is then preferentially, although not exclusively, reduced to malate or aminated to aspartate. This occurs in the mesophyll cells; the two dicarboxylates are then transferred to the bundle sheath cells to undergo decarboxylation, yielding carbon dioxide which is refixed by the Calvin cycle occurring in the bundle sheath cells. The remaining C₃ compound, alanine, pyruvate or phosphoenol pyruvate, returns to the mesophyll cells (Rathnam, 1978).

Within the bundle sheath cells the pathway of decarboxylation varies depending on the plant species involved. The three different pathways depend on the decarboxylating enzymes utilised and are shown in Fig. 1.12 (Rathnam and Chollet, 1980). As can be seen both an aspartate aminotransferase and an alanine aminotransferase are involved in all three reaction pathways. The mitochondrial aspartate aminotransferase deaminates aspartate to oxaloacetate, which is then decarboxylated to malate, with the carbon dioxide entering the Calvin cycle. The role of the cytoplasmically located alanine aminotransferase is
Fig. 1.11. The Carbon Shuttle in C₄ Plants.

The movement of recently fixed carbon (C) from mesophyll cells to bundle sheath cells, where the CO₂ is regenerated and enters the Calvin cycle again. The C₃ carrier compound is returned to the mesophyll cells for reuse.
Fig. 1.12. The Carbon Shuttle in Bundle Sheath Cells of the Three Types of C₄ Plants.

Three different types of C₄ plants have evolved and are named by the main enzyme used for decarboxylating the C₄ compound in bundle sheath cells:

(a) NADP dependent malic enzyme type
(b) NAD dependent malic enzyme type and
(c) Phosphoenolpyruvate carboxykinase type.

The major route of the metabolites is shown with dark arrows and the compounds are written in bold face; other routes which occur in the type of C₄ plant are also shown.
From MC → Aspartate
To MC → Alanine
From MC → Malate
To MC → Pyruvate

Mitochondrion

Chloroplast
(b)

From HC -- Aspartate
To MC -- Alanine

Aspartate

Kg

Glu

OAA

Pyruvate

Pyruvate

NADH

NAD^+

Malate

Mitochondrion

CO_2

From MC -- Malate

Malate

GAP

NAD(P)H

NAD(P)^+

PGA

RuBP

Chloroplast
From MC → Aspartate → Aspartate
To MC ← Alanine

From MC → Malate → Malate
To MC ← PEP

Pyruvate → Pyruvate

OAA → NADH → NAD⁺ → Malate

Mitochondrion

Malate

OAA

PGA

GAP

NAD(P)H

NAD(P)⁺

CO₂

RuBP

Chloroplast
to regenerate 2-oxoglutarate for the aspartate aminotransferase. This aminotransferase cycle has been verified by the equal amounts of increase in aspartate decarboxylation by the addition of either 2-oxoglutarate or pyruvate (Rathnam and Edwards, 1977).

With the important role that aminotransferases play in nitrogen and carbon metabolism, changes in aminotransferase systems with plant and organ development will obviously have important effects on that development. Many investigators (reviewed in Wightman and Forest, 1978) have shown that aspartate and alanine aminotransferase activities increase markedly in the first few days of germination, then reach a peak and declined thereafter. The time taken to reach the peak activity depended on the organ investigated. In *Phaseolus vulgaris*, the cotyledons reached peak activity after one day, the shoots in eight days and the roots in ten days (Forest and Wightman, 1972a).

Some changes in activity, particularly increases, could be linked to specific developmental changes. Hedley and Stoddart (1972) related leaf emergence and expansion to an increase in alanine aminotransferase activity. Hatch and Mau (1973) demonstrated a tenfold increase in aspartate and alanine aminotransferase activity during greening of the leaves of the C₄ plant *Panicum milaceum*, obviously required for the carbon shuttle.

Environmental effects have also been shown to cause changes in the amount of aminotransferase activity, especially the effects of photoperiod. Under short day conditions, the levels of alanine and aspartate aminotransferase activity in *Phaseolus* seedlings remained at the peak activity, while in long day conditions they declined (Forest and Wightman, 1972a), although gibberellin could maintain the alanine aminotransferase activity at peak levels in long day conditions (Hedley and Stoddart, 1971).
The method of control of aminotransferase levels in plants is almost completely unknown, although Pilet (1971) has shown that in *Lens culinaris* root cells aminotransferase activity decreases with abscisic acid application, and kinetin counteracts the abscisic acid effect. There are suggestions that the appearance of alanine aminotransferase isoforms in developing plants is light controlled (Otter *et al.*, 1992). This effect of light seems to be mediated by the photochrome system, which means that the only isoforms regulated are chloroplast located, the cytosolic enzymes being independent of light effects (Penther, 1991).

However the control of animal tyrosine aminotransferase is well documented (Hargrove and Granner, 1985). Levels are controlled by alterations in the rate of synthesis and degradation of the enzyme. Various hormones, including insulin, glucagon and hydrocortisone, increase synthesis of the aminotransferase by increasing mRNA transcription and stabilising existing mRNA. Degradation of the enzyme is controlled by a number of metabolic factors including low amino acid concentrations, especially aromatic ones. These factors combine to cause changes in the amount of tyrosine aminotransferase over the course of a day, in response to various stimuli, usually associated with feeding and fasting.

1.4.2 Properties

Animal aminotransferases have been purified to homogeneity and extensively studied, with the complete amino acid sequence and quaternary structure of some enzymes have been determined (Christen and Metzler, 1985). The most extensively studied of these is aspartate aminotransferase, which exists as cytosolic and mitochondrial
isoenzymes. It consists of two identical subunits each with a molecular mass of about 48,000. Each subunit has an active site containing the covalently bound coenzyme pyridoxal phosphate.

Few plant aminotransferases have been fully purified, but studies on partially purified enzymes show they have many properties in common with animal aminotransferases (Table 1.2). Molecular weights fall into three major groups: a monomeric 40-60 kDa group (Roberts, 1977; Reynolds, 1991), an 80-120 kDa group, almost certainly dimeric, though not necessarily identical (Griffith and Vance, 1969; Verjee and Evered, 1969; Reynolds et al., 1981). The third group all contain four subunits, again not necessarily identical, with molecular weights varying between 170 and 290 kDa (Prasad, 1989; De-Eknumkul and Ellis, 1987).

Most plant aminotransferases show a pH optimum of around 8.0 (Forest and Wightman, 1972; Wink and Hartmann, 1981). There is evidence from inhibition results and quantitative determination that plant aminotransferases contain pyridoxal phosphate (Wong and Cossins, 1969; Roberts, 1977, Lu and Mazelis, 1975). In animals this coenzyme dissociates from the enzyme during purification to give the apoenzyme, free of pyridoxal phosphate. In contrast, plant aminotransferases frequently do not lose the pyridoxal phosphate during purification, and hence are either marginally or not activated by the addition of pyridoxal phosphate to the assay medium (Reed and Hess, 1975; Rech and Crouzet, 1974).

For most plant aminotransferases the $K_m$ for the oxo-acid substrate is lower than that of the amino acid substrate (Reynolds et al., 1981). The observed $K_m$ values may depend on the conditions of the assay as is true for animal aminotransferases (Braunstein, 1973). The amino acid $K_m$ values seem very high, usually between 0.2 and 0.6 mM, but this may be reasonable considering the levels of amino acids in some
<table>
<thead>
<tr>
<th>Type</th>
<th>Source</th>
<th>Km</th>
<th>MW</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate: Oxoglutarate</td>
<td>Oat leaf</td>
<td>4.14</td>
<td>130</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reed and Hess (1975)</td>
</tr>
<tr>
<td>Aspartate: Oxoglutarate</td>
<td><em>Panicum maximum</em></td>
<td>2.3</td>
<td>100</td>
<td>2x42</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Numazawa <em>et al</em> (1989)</td>
</tr>
<tr>
<td>Aspartate: Prephenate</td>
<td><em>Anchusa officinalis</em></td>
<td>0.08</td>
<td>220</td>
<td>2x44, 2x57</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>de Eknamkul and Ellis (1988)</td>
</tr>
<tr>
<td>Alanine: Oxoglutarate</td>
<td>Tomato mitochondria</td>
<td>2.5</td>
<td>100</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Gazeu-Reyjal and Crouzet (1976)</td>
</tr>
<tr>
<td>Alanine: Oxoglutarate</td>
<td>Pumpkin cotyledon</td>
<td>1.70</td>
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<td>nd</td>
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<td></td>
<td></td>
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<td>Splittstoesser <em>et al.</em> (1976)</td>
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Table 1.2. Plant Aminotransferases.
<table>
<thead>
<tr>
<th>Type</th>
<th>Source</th>
<th>Km</th>
<th>mM</th>
<th>MW</th>
<th>kDa</th>
<th>Reference</th>
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<tr>
<td>Alanine: Oxoglutarate</td>
<td>Panicum millaceum</td>
<td>6.67</td>
<td>0.15</td>
<td>102</td>
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<td>Son et al. (1991)</td>
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<tr>
<td>Alanine: Oxooctanol</td>
<td>Conium maculatum</td>
<td>27.0</td>
<td>0.14</td>
<td>56</td>
<td>nd</td>
<td>Roberts (1977)</td>
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<tr>
<td>Alanine: 4,5-dioxovaleric acid</td>
<td>Pennisetum typhoideum</td>
<td>3.4</td>
<td>1.8</td>
<td>168</td>
<td>4x42</td>
<td>Prasad and Prasad (1989)</td>
</tr>
<tr>
<td>Serine: Glyoxylate</td>
<td>Kidney bean leaf</td>
<td>0.71</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>Smith (1973)</td>
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<td>Serine: Glyoxylate</td>
<td>Spinach leaf</td>
<td>2.72</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>Rehfeld and Tolbert (1972)</td>
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<tr>
<td>Serine: Glyoxylate</td>
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<td>91</td>
<td>2x43</td>
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<td>Paszkowski and Niedzirksa (1990)</td>
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Table 1.2, continued. Plant Aminotransferases.
<table>
<thead>
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<th>Reference</th>
<th>Type</th>
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<th>MW</th>
<th>Km</th>
<th>Reference</th>
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<td>Chapter et al. (1990)</td>
<td>Bradyrhizobium</td>
<td>Oxidoreductase</td>
<td>Oxo-isovalerate</td>
<td>0.4</td>
<td>0.02</td>
<td>0.1</td>
<td>3</td>
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<td>Amares (1991)</td>
<td>Melilotus</td>
<td>Oxidoreductase</td>
<td>Oxo-3-methyl</td>
<td>6.3</td>
<td>5</td>
<td>2.5</td>
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<tr>
<td>Singh et al. (1991)</td>
<td>Melilotus</td>
<td>Oxidoreductase</td>
<td>Oxo-isovalerate</td>
<td>0.25</td>
<td>0.25</td>
<td>2.5</td>
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Table 1.2. Continued. Plant Aminotransferases.
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<th>Type</th>
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<th>MW</th>
<th>kDa</th>
<th>Subunit</th>
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<td>Glyoxylate</td>
<td>Glutamate</td>
<td>2.6</td>
<td>0.5</td>
<td>58.8</td>
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<td>One</td>
<td>Paszkowski and Niedzieska (1989)</td>
</tr>
<tr>
<td></td>
<td>Glyoxylate</td>
<td></td>
<td></td>
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<tr>
<td>Asparagine</td>
<td>Pea leaf</td>
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<td>Asparagine</td>
<td>3.9</td>
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<td>105</td>
<td>nd</td>
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<td>Ireland and Joy (1983)</td>
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<td></td>
<td>Serine</td>
<td></td>
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<td></td>
<td>Pyruvate</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Tyrosine:</td>
<td><em>Anchusa officinalis</em></td>
<td></td>
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<tr>
<td>Oxoglutarate</td>
<td>Tyrosine</td>
<td>0.5</td>
<td></td>
<td>220</td>
<td>4x56</td>
<td></td>
<td>de-Eknamkul and Ellis (1987)</td>
</tr>
<tr>
<td>Tyrosine:</td>
<td><em>Anchusa officinalis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxoglutarate</td>
<td>Tyrosine</td>
<td>2.0</td>
<td></td>
<td>180</td>
<td>4x43</td>
<td></td>
<td>de-Eknamkul and Ellis (1987)</td>
</tr>
</tbody>
</table>

Table 1.2, continued. Plant Aminotransferases.

nd = not determined
organelles is up to 16mM (Mills and Joy, 1980; Chapman and Leech, 1979). The accepted reaction mechanism is ping pong bi bi binary (Wong and Cossins, 1969; Broke et al., 1970; Ryan et al., 1972; Forest and Wightman, 1973; Roberts, 1977). This is the same as animal aminotransferases, and since both plant and animal aminotransferases require pyridoxal phosphate as a cofactor the reaction mechanisms are probably identical.

The mechanism for animal aspartate aminotransferase is well characterised (Christen and Metzler, 1985) and consists of two half reactions of eight steps (Fig.1.13). The amino acid substrate binds to the pyridoxal phosphate form of the enzyme then undergoes a condensation reaction with the pyridoxal phosphate to give an aldimine. This rearranges to give a ketimine which is then hydrolysed to give the oxo acid plus the enzyme in the pyridoxamine form. The pyridoxal phosphate form of the enzyme is regenerated by the reverse reaction using the oxo acid substrate. Substrate affinity is determined by the enzyme structure around the active site; because few plant aminotransferases have been purified to homogeneity it is difficult to give definite conclusions on substrate specificities. Highly purified aminotransferases seem to be multispecific: bush bean aspartate aminotransferase was able to transaminate phenylalanine, tryptophan and tyrosine as well as aspartate, although at much reduced activities (Forest and Wightman, 1972). Generally aminotransferases exist with differing substrate specificities and activities which make characterisation difficult. For example, a tryptophan and phenylalanine aminotransferase from liver mitochondria was found to be the aspartate:oxaloacetate aminotransferase (Miller and Littweck, 1971).
Fig. 1.13. Aspartate Aminotransferase Mechanism.

(1) Binding of the amino acid substrate to the nonprotonate pyridoxal phosphate form of the enzyme, yielding the Michaelis complex.

(2) Transfer of a proton from the amino group of the substrate to the aldimine nitrogen atom of the cofactor.

(3) Initiation of the transaldimination stage: displacement, via a tetrahedral intermediate of the lysine ε-amine group from the internal Schiff base.

(4) Completion of transaldimination stage: Intermediate proton transfer, forming an 'external' pyridoxal phosphate-substrate aldimine.

(5) Dissociation of the α-hydrogen from the aldimine, forming a quinoid intermediate.

(6) Protonation of the quinoid intermediate to form the pyridoxamine phosphate cofactor.

(7) Hydrolysis of the ketimine.

(8) Release of the product oxo acid, with the formation of the pyridoxamine enzyme complex.
Aspartate 1

Michaelis complex

Geminal diamine

External aldimine

Quinoid intermediate

Ketimine

Oxaloacetate

Pyridoxamine phosphate form
1.4.3 Tryptophan Aminotransferase

Due to the apparent multispecificity of aminotransferases, tryptophan aminotransferases are usually considered as part of the group of aromatic aminotransferases. Unfortunately due to the difficulty of measuring activity with tryptophan, many so-called aromatic aminotransferases have been shown to be active only with tyrosine and phenylalanine. However, in bacteria aromatic aminotransferases with tryptophan activity have been found in *E. coli* (Mavrides and Orr, 1975), *Brevibacterium linens* (Lee and Desmazeaud, 1985), *Rhizobium leguminosarum* (Perez-Galdina *et al.*, 1989) and *Festuca octoflora* (Frankenburger and Poth, 1988), all of which have a pH maximum around 8.0, and where characterised contained two subunits, each with molecular weight of 44,000 and tightly bound pyridoxal phosphate.

The first thorough investigation of tryptophan aminotransferase in plants was carried out by Truelson (1972, 1973), who purified a mung bean tryptophan aminotransferase 27 fold. Despite being called a tryptophan aminotransferase, the enzyme showed greater activity with the other aromatic amino acids and with alanine, leucine, lysine, methionine and arginine, although D-amino acids were not transaminated. 2-Oxoglutarate, oxaloacetate and pyruvic acid all were able to be used as the amino group acceptor. Further investigation (Truelson, 1973) showed that this enzyme was distributed widely throughout the plant kingdom, being found in plants in 16 different families, including some species of algae.

At a similar time, an aromatic aminotransferase was isolated from bushbean seedlings (Forest and Wightman, 1972a,b). This enzyme was purified 600 fold by pH and ammonium sulfate precipitation, gel filtration and DEAE anion exchange to give a homogeneous protein as
assessed by by PAGE. This enzyme showed activity only with the L forms of the aromatic amino acids, asparagine and aspartic acid, however the activity with the latter was more than ten times greater than with the aromatic substrates.

Another form of aromatic aminotransferase was purified by Noguchi and Hayashi (1980), once again this was multispecific with highest activity with serine and alanine, but unlike the previous two enzymes the only suitable amino acceptors were glyoxylate and hydroxypyruvate. Two isoenzymes were shown to exist, one in the cytoplasm and the other in the peroxisomes.

Compartmentation by differential centrifugation also showed that there were two or more isoenzymes of the enzyme isolated by Truelson (1972); one of these was cytoplasmic and the other associated with nonchloroplast organelles (Vackova et al., 1985).

Further investigation of these enzymes have involved comparing enzyme activity with tryptophan and indoleacetic acid content in normal and tumourous tobacco tissue cultures (El Bahr et al., 1987). There seems to be little correlation between the activity of the enzyme, which gradually increased and changes in indoleacetic acid and tryptophan content. However, a change in the pH optimum from 8.5 in normal tissue to 9.3 in the tumourous tissue was observed.

A D-tryptophan aminotransferase has also been isolated (McQueen-Mason & Hamilton, 1989) which is located in plastids. This was purified 1,500 times and was shown to have no activity with L-tryptophan, although its specificity with other D-amino acids was not determined.
1.5 Aims of this Thesis

The role of tryptophan aminotransferase in control of indoleacetic acid synthesis was studied since the aminotransferase is the initial enzyme in the putative pathway of indoleacetic acid biosynthesis, and thus the most likely to be involved in any feedback inhibition. Additionally, there is proof of changes in the activity of tryptophan aminotransferase in comparing auxin heterotrophic and autotrophic tissue cultures of Nicotiana tabacum (Gaal and Koves, 1981); the autotrophic cultures had markedly higher tryptophan aminotransferase activities.

Cell suspension cultures were used as the basis for the experiments because they provide a long term source of material, which develops in the same manner over each subculturing of the cultures. Mousdale (1982) has shown that as the cultures develop, the amounts of indoleacetic acid change in a regular fashion, making the cultures an ideal tissue source to study the changes in indoleacetic acid and its precursors and biosynthetic enzymes.

The aim of this thesis was to discover what, if any, role tryptophan aminotransferase played in the control of biosynthesis of indoleacetic acid. To achieve this, several lines of experimentation were attempted. These were looking at indoleacetic acid synthesis in cell free extracts, purification, characterisation and partial sequencing of tryptophan aminotransferase, and measuring the levels of indoleacetic acid, indolepyruvate and tryptophan over the development of the suspension cultures.
2. INDOLEACETIC ACID PRODUCTION IN CELLFREE EXTRACTS

2.1. Introduction

The usual method of determining whether putative precursors of indoleacetic acid are involved in its formation is by the exogenous application of the precursor labelled with a radioactive isotope. An example of this was the use of carbon-14 labelled tryptophan by Dannenburg and Livermann (1957) which yielded the first concrete evidence that tryptophan was a precursor to indoleacetic acid.

However there are major drawbacks with this method. It is completely dependent on the uptake of the precursor into the cell and its equilibration within the various compartments of the plant cell, especially that compartment in which conversion to indoleacetic acid takes place. This also leads to dilution of the labelled compound, with compounds like tryptophan being mostly sequestered to the vacuole. For example, the uptake of 5-3H-tryptophan into Pinus sylvestris (Scots pine) protoplasts was only 5%-10% of that applied (Sandburg et al., 1990). This is further complicated by the fact that the fate of exogenously applied precursor may well be different from the normal metabolism of the compound. This atypical metabolism may be caused by far higher than normal levels of the compound, or by entry of the precursor into cellular pools in which it is not usually found.

An alternative method, also utilising labelled compounds, involves equilibrating the plant or tissue with deuterium oxide (D₂O). D₂O distributes throughout all cellular pools without qualitatively altering
plant metabolism (Mitra et al., 1976), and the deuterium may be incorporated into various compounds through both de novo synthesis and hydrogen exchange (Pengelly et al., 1983). The role of various compounds as precursors to indoleacetic acid can then be checked by monitoring the incorporation of the deuterium label into the precursor and the indoleacetic acid (Cooney and Nonhebel, 1991a). This method has to be used with care due to problems caused by the isotope effect of deuterium, which include slow development and, at high concentrations, toxicity.

In contrast to these methods of studying the biosynthesis of indoleacetic acid in whole tissue with labelled compounds, the biosynthesis may also be studied using cell free extracts of tissues. This method has been used to prove the involvement of an aminotransferase in the synthesis of indoleacetic acid in Rhizobium leguminosarum biovar Trifolii (Perez-Galdona et al., 1989) by showing the requirement for 2-oxoglutaric acid and pyridoxal phosphate, cofactors for an aminotransferase, and also showing that O2 and NAD+, cofactors for dehydrogenases, were not required.

The biosynthesis of indoleacetic acid from both L- and D-tryptophan has also been demonstrated in crude extracts of pea seedlings (McQueen-Mason and Hamilton, 1989), showing the requirement for NAD+, pyruvate and, to a lesser extent, pyridoxal phosphate.

Thus, using a cellfree extract, it is possible to measure indoleacetic acid synthesis from various precursors. This method has a major disadvantage, in that the cells and the contained organelles has been lysed, so it is possible that enzymes which are usually not found in the same compartment are able to associate, yielding a pathway not found in vivo. However these effects may be monitored and removed by
separating the various cell organelles using centrifugation, and repeating the experiments on each organelle type. This also makes it possible to determine which organelle is involved in indoleacetic acid biosynthesis.

The method is also very useful in the ease in which various cofactors, inhibitors and substrates may be added. The possibility of an intermediate, such as indolepyruvate, can be ascertained by cold trapping. For example, if cold indolepyruvate, as well as radiolabelled tryptophan was added to an homogenate and incubated, there would be less radiolabelled indoleacetic acid formed than that formed by an incubation with no added indolepyruvate. This is because with the added indolepyruvate, labelled indolepyruvate would be diluted by the large amount of unlabelled indolepyruvate so the chance of being converted to indoleacetic acid would be less, hence less labelled indoleacetic acid.

Multienzyme complexes can be investigated using this system. If $^{14}$C-labelled tryptophan and $^{3}$H-labelled indolepyruvate were incubated together, then the amount of tritium and carbon-14 labelling of the formed indoleacetic acid would be indicative of the freedom of the indolepyruvate to both diffuse from the enzyme that formed it and become the substrate of the next enzyme in the pathway. This would reveal any enzyme complexes similar to that of the ent-kaurene synthetase involved in GA synthesis (Duncan and West, 1981).

The role of cofactors in the biosynthesis of indoleacetic acid is very important, especially as different enzymes able to catalyse the same reaction use different cofactors. For example the two types of enzymes forming indolepyruvate from tryptophan, aminotransferases and dehydrogenases, differ markedly in requirements: dehydrogenases require NAD$^+$ or NADP$^+$, while aminotransferases require an oxo acid acceptor group, and possibly pyridoxal phosphate. Indeed, various
aminotransferases have differing specificities to different oxo acids. For instance, Truelson (1972) isolated a tryptophan aminotransferase with most activity using pyruvate, oxalacetate and 2-oxoglutaric acid, and little activity with glyoxylic acid and hydroxypyruvate, while Noguchi and Hayashi (1980) isolated one with exactly the opposite specificities.

Other cofactors which have been suggested to be required by various putative enzymes of indoleacetic acid biosynthesis include NAD+, which can be used by two possible enzymes of indoleacetic acid biosynthesis: indoleacetaldehyde dehydrogenase which converts indoleacetaldehyde to indoleacetic acid (Asker and Davies, 1985), and tryptophan dehydrogenase, which forms indolepyruvate from tryptophan (Kutacek, 1985). Cysteine has been shown to be required for indoleacetaldehyde oxidase activity (Rajagopal, 1971) converting indoleacetaldehyde to indoleacetic acid. Cocarboxylase (thiamine pyrophosphate chloride) was also added as a cofactor for the putative enzyme for the conversion of indolepyruvate to indoleacetaldehyde: indolepyruvate decarboxylase.
2.2. Materials and Methods

2.2.1. Materials

Chemicals

With the exception of the chemicals given below, all the chemicals used in these experiments were purchased from Sigma Chemical Company at Analar grade.

DL-[methylene-\textsuperscript{14}C]Tryptophan, 1.9GBq/mmol from Amersham International.

L-\textsuperscript{[5-3H]}Tryptophan, 0.9TBq/mmol from Amersham International

Methanol: ChromAR HPLC grade from Mallinckrodt

Plant Material

Mung beans (\textit{Vigna radiata}) were purchased from Woolworths Ltd. They were soaked for 16 hours prior to sowing on water saturated vermiculite. The beans were grown in the dark at approximately 20\textdegree C.

Columns

Sepralyte \textsubscript{C\textsubscript{18}} Column: 5mL of preparative grade Sepralyte \textsubscript{C\textsubscript{18}} from Analytichem International packed in a 1cm diameter column.
L-Proline chiral HPLC column: prepared by the method of Grierson and Adams (1985), from a Brownlee silica Speri-5 column (4.6mm id x 100mm).

Polymer Reverse Phase (PRP) HPLC column: Polymer RP (4.6mm id x 100mm, 10μm particle size) from Brownlee.

2.2.2. Resolution of DL-Tryptophan

Chiral buffer: 50mM KH₂PO₄, 1mM CuSO₄, made up 500mL at a time, with the pH adjusted to 4.00 using conc. HCl.

TEA solution: 2% acetic acid, adjusted to pH 3.5 using triethylamine

The chiral column was equilibrated with chiral buffer at 0.5mL per minute for at least an hour, then the separation was checked using a DL tryptophan mixture. Retention times were approximately 8 and 3.5 min for L and D respectively (see Fig. 2.1).

400μL of ¹⁴C-tryptophan (approximately 5.6x10⁶ DPM) were dried down under nitrogen in a silanised vial and then redissolved in 200μL of chiral buffer. This was applied to the chiral column, and the two enantiomers were collected separately. The D-tryptophan fraction was applied to the column again, since its retention time was close to the void volume of the column and it was possibly contaminated.

Each of the D and L fractions were then diluted with 20mL of TEA solution, and applied to the Sepralyte C₁₈ column, which had been equilibrated with TEA solution. The column was washed with 5mL of TEA solution, then the tryptophan eluted with 10mL methanol.
Fig. 2.1. Resolution of D and L-Tryptophan by L-Proline Chiral Column.

The chiral column, prepared by the method of Grierson and Adam (1985), was equilibrated with pH4.0 50mM KH$_2$PO$_4$, 1mM CuSO$_4$ at 0.5mL/min for an hour prior to the application of a sample containing approximately 10μg each of D-tryptophan and L-tryptophan. The retention times were 3.5 minutes for D-tryptophan and 8 minutes for L-tryptophan.
Samples were dried down under nitrogen and redissolved in 50% isopropanol and stored at -20°C until needed. Yields were approximately 70% for L-tryptophan and 60% for D-tryptophan.

2.2.3. Separation of Indoleacetic Acid, Tryptophan and Indolepyruvate Mixtures

TEA solution: 2% acetic acid, adjusted to pH 3.5 using triethylamine.
Aqueous acetic acid: pH 2.5 acetic acid in milliQ water, approximately 0.25% acetic acid.

Polymer Reverse Phase Column

The HPLC system used in these experiments was Waters Model 501 and 510 pumps, controlled by a Waters Automated Gradient Controller. The detector used was a Waters Model 441 Absorbance Detector, set at 280nm. The best separation for standard mixtures was found to be using an isocratic solvent of 70% methanol and 30% aqueous acetic acid at a flowrate of 0.7mL/min.

The retention times were 1.8min for tryptophan, 4.4 min for indoleacetic acid and 7.7 min for indolepyruvate, a typical trace is given in Fig 2.2. The overlap between the tryptophan and indoleacetic acid peaks was checked using both radiolabelled tryptophan and indoleacetic acid, similarly the overlap between the indoleacetic acid peak and the indolepyruvate peak was checked using radiolabelled indoleacetic acid, and in all cases there was full resolution between the peaks. It was not possible to investigate the coelution of indolepyruvate with the
Fig. 2.2. Separation of Tryptophan, Indoleacetic Acid and Indolepyruvate on Polymer Reverse Phase.

Conditions are as given in 2.2.3; solvent was 70% methanol, 30% pH2.5 aqueous acetic acid, flowing at 0.7mL/min. Approximately 10μg of each of the compounds was applied as a mixture to the column. Retention times are 1.8min for tryptophan, 4.4 min for indoleacetic acid and 7.7min for indolepyruvate.
indoleacetic acid peak because of the lack of a fully pure radiolabelled indolepyruvate standard.

While using the PRP column for separation of standard mixtures gave good reproducible results, using the column with crude plant samples gave problems with drifting retention times, overloading of the column and in some cases column overpressure, despite using a precolumn. Hence a method for crude fractionation of the plant samples was required before using the HPLC.

**Sepralyte C\_18**

Investigation showed that when mixtures of standard indoleacetic acid, indolepyruvate and tryptophan were applied to the Sepralyte C\_18 column, which had been equilibrated with aqueous acetic acid, then the three compounds could be crudely separated using a gradient of methanol in aqueous acetic acid. Tryptophan was released by 40% methanol, then indoleacetic acid with 50% methanol and then finally indolepyruvate at 70% methanol.

The separation was not as well resolved as that using the PRP column, approximately 5% of the indoleacetic acid came off in the tryptophan fraction, although negligible tryptophan remained to elute with the indoleacetic acid fraction. There was also a sizable fraction of indolepyruvate, between 15 and 20%, in the indoleacetic acid peak, as determined by spectrophotometry.

This made separation on the Sepralyte C\_18 suitable for crude results only. However, a combination of this column and the PRP column was found to give excellent reproducible results. The Sepralyte C\_18 column was used to separate the tryptophan, and most of the rest of the crude
sample from the indoleacetic acid and indolepyruvate, by applying the sample, eluting the tryptophan with 40% methanol in aqueous acetic acid, then eluting the indoleacetic acid and indolepyruvate with 70% methanol in aqueous acetic acid. A fraction of this second sample was then applied to the PRP without compromising the separation of the compounds.

### 2.2.4. Determination of Indoleacetic Acid and Indolepyruvate Synthesis in Cellfree Extracts

Extraction buffer: 50mM phosphate, 1mM MnCl₂, 2mM EDTA, 50mM isoascorbic acid, pH 8.5.

Incubation buffer base: 50mM phosphate, 1mM MnCl₂, 2mM EDTA, pH 8.5.

Internal standard solution: 1mg/ml indoleacetic acid, 1mg/ml indolepyruvate, 1mg/ml BHT in methanol.

Scintillation cocktail: 1.0L toluene, 0.5L Triton X-100, 4.0g PPO

Plant Extraction.

All steps in extraction of the mung beans took place at 40°C. Approximately 20g of freshly harvested mung beans grown for six to eight days at 25°C in the dark were used in each extraction. The crude extract was prepared by homogenising the beans in one volume of extraction buffer, along with polyvinylpyrrolidone in a 1:0.05 ratio with the tissue. The mixture was homogenised for at least a minute at
full speed, then the extract expressed through four layers of cheesecloth. The residue was reextracted with another half volume of extraction buffer and expressed through cheesecloth. The combined filtrates were then spun for 20 minutes at 27,000g. the resulting supernatant was decanted off and kept on ice in the dark until used, and not for longer than six hours. Any extracts that were, or became, markedly browning were discarded.

**Incubation**

The incubation mixture consisted of 0.9mL of the mung bean extract and 0.1mL of the incubation buffer. The latter consisted of the incubation buffer base with the addition of various cofactors and substrates. The cofactors used varied from experiment to experiment, but were taken from the following list with the final concentrations in the incubation mixture as given in Table 2.1.

<table>
<thead>
<tr>
<th>Cofactor or Substrate</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>D- or L- Tryptophan</td>
<td>1mM</td>
</tr>
<tr>
<td>Pyridoxal phosphate</td>
<td>10μM</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>1mM</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>1mM</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>1.25mM</td>
</tr>
<tr>
<td>Cocarboxylase</td>
<td>0.1mM</td>
</tr>
<tr>
<td>Cysteine-HCl</td>
<td>0.1mM</td>
</tr>
<tr>
<td>Indolepyruvate</td>
<td>10mM</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>5mM</td>
</tr>
</tbody>
</table>

Table 2.1 Final Substrate and Cofactor Concentrations Used in the Incubation of Cellfree Extract Mixtures.
The assay mixture also contained either $[^3\text{H}]-\text{L-tryptophan}$ with a specific activity of 150,000 DPM or $[^{14}\text{C}]-\text{D-tryptophan}$ with a specific activity of 300,000 DPM, depending on which enantiomer was being used for the incubation. Controls consisted of the same incubation buffer, but used extract that had been boiled for two minutes to remove enzyme activity.

The incubation consisted of a five minute preincubation of the extract at $40^\circ\text{C}$ in an Eppendorf tube, followed by the addition of the radiolabelled tryptophan and the incubation buffer to initiate the reactions. Incubation was carried out at $40^\circ\text{C}$ for an hour and was terminated by the addition of 0.1mL of glacial acetic acid and 0.1mL of the internal standard mix. As well as acting as an internal standard this latter mixture also helped to stabilise the indoleacetic acid and indolepyruvate both by the presence of the antioxidant BHT and by the large amounts of unlabelled compound, diluting the effects of any losses.

**Indoleacetic Acid and Indolepyruvate Measurements**

The periods of incubation were started at staggered intervals to enable all the following steps to be carried out without any delays so as to decrease the amount of product breakdown.

Directly after incubation the mixture was spun at 13,000xg for five minutes to sediment any precipitate, and then applied to the Sepralyte C$_{18}$ column, which was pre-equilibrated with aqueous acetic acid. The column was washed with 5mM of aqueous acetic acid, and then the tryptophan eluted with 10mL of 40% methanol in aqueous acetic acid. The indoleacetic acid and indolepyruvate were eluted simultaneously using 10mL of 70% methanol.
While the assay mixture was being spun and separated on the Sepralyte C18 column an aliquot of the internal standard mixture containing a similar amount of indoleacetic acid and indolepyruvate as expected from the sample (about 5μL of the internal standard mixture) was injected onto the HPLC to check retention times and amounts of the two compounds. The HPLC was set up as given in Section 2.2.3: using the PRP column with a solvent of 70% methanol; 30% aqueous acetic acid flowing at 0.7mL/min.

Immediately the indoleacetic acid/indolepyruvate fraction eluted from the Sepralyte C18 column, 500μL of it was injected onto the HPLC for further separation. The HPLC chromatograms were analysed by version 3.11 of the Delta Junior package from Digital Solutions Ltd to measure the peak areas. The amount of radioactivity in 100μL aliquots of the Sepralyte C18 fractions and 200μL aliquots of the HPLC fractions were measured by liquid scintillation in a LKB 1209 Rackbeta Liquid Scintillation Counter, using 4mL per vial of scintillation cocktail. The counting efficiency was determined by external standard.

Analysis of Results

The first step in determining the conversion of tryptophan to indoleacetic acid and indolepyruvate was to quantitate the amount of each compound in the relevant fraction of the HPLC separation. This was carried out using the internal standards and also a calibration curve for each of the compounds, which had been constructed earlier. It was also assumed that all the indolepyruvate and indoleacetic acid seen in the HPLC peaks was from the internal standard (i.e. indoleacetic acid and indolepyruvate synthesis were negligible compared to the addition
of 0.1mg of each). Thus the losses of indoleacetic acid and indolepyruvate between addition of the internal standard and quantification on the HPLC could be determined.

The true amount of labelled indolepyruvate and indoleacetic acid were then determined, using the activity in the HPLC fraction, and the calculated losses, and then conversion to the compounds from tryptophan was computed as a percentage of tryptophan added to the assay mixture.
2.3. Results and Discussion

The results of two typical experiments can be seen in Figure 2.3. The cofactors used in these experiments were NAD⁺, pyruvate, 2-oxoglutaric acid, cysteine, cocarboxylase and pyridoxal phosphate at the concentrations given in Table 2.1. All these cofactors were used in an attempt to get some formation of indoleacetic acid and indolepyruvate, but, as can be seen in the figure, there was no significant difference between the incubations containing unboiled extract and those containing boiled extract. In all cases the sample and the blank were within the standard error of each other, and were frequently the same or very close. This suggests that the only conversion of tryptophan into indoleacetic acid and indolepyruvate under the experimental conditions used was by nonspecific reaction.

The most obvious reason for this lack of production of indoleacetic acid and indolepyruvate is that the enzymes involved do not have sufficient turnover to produce a significant result. Nonhebel and Cooney (1990) measured the turnover of indoleacetic acid in the intact cell as 3.6nmol/hour/g fresh weight, a turnover too low to be measured by the experimental method used. This is belied by the fact that McQueen-Mason and Hamilton (1989) were able to measure the formation of indoleacetic acid in pea seedlings.

These comparisons should considered with some doubt; they are comparing different plants, and in the case of the data from Nonhebel and Cooney (1990) the turnover is measured over the whole shoot, while most of the indoleacetic acid biosynthesis probably occurs in the
Fig. 2.3. Conversion of Tryptophan to Indoleacetic Acid and Indolepyruvate in Cellfree Extracts.

Each bar on the graph is the average of three measurements of the compound using the same extract, and the error plotted is the standard deviation of the three values divide by the square root of the number of measurements.

Both the experiments were carried out under identical conditions, differing only in that they used different plant extracts. The incubation buffer used was 50mM phosphate, 1mM MnCl$_2$, 2mM EDTA, 0.1mM pyridoxal phosphate, 1mM 2-oxoglutarate, 1mM pyruvate, 12.5mM NAD$^+$, 10mM cocarboxylase, 10mM cysteine and 1mM tryptophan, either D or L, as described in 2.2.4. Additionally, either 150,000 DPM of $^{3}$H-L-tryptophan or 300,000 DPM of $^{14}$C-D-tryptophan was added to the incubation, depending on the enantiomer used. The mixture was incubated for an hour at 40°C, then stopped by the addition of glacial acetic acid, followed by 0.1mg of indoleacetic acid and 0.1mg of indolepyruvate. The indoleacetic acid and indolepyruvate were separated and quantitated by Sepralyte C$_{18}$ and PRP columns as described in 2.2.3 and 2.2.4.
Conversion of Tryptophan in Cellfree Extracts

- IAA in Sample
- IAA in Blank
- IPyA in Sample
- IPyA in Blank

Percentage Conversion

L-trp  D-trp  L-trp  D-trp
shoot apex, where the turnover would be much greater. Further, prior to beginning the experiments, the expected turnover was calculated using the results of Truelson (1972) who investigated the formation of indolepyruvate in extracts of mung bean shoot apexes.

Truelson (1972) measured a conversion of tryptophan to indolepyruvate at a rate of 8.3 nmol/min/g of shoots. The incubations used in these experiments used approximately half a gram of tissue, and were incubated for an hour. Hence the expected production of indolepyruvate would be about 250 nmol. Each assay also started with 1 µmol of tryptophan, thus conversion and beyond should be around 25% or a slightly less due to decreased saturation of the enzymes. This result is obviously far in excess of the approximately 3-4% incorporations measured in both blank and sample.

These calculations suggest, therefore, that it is not a lack of potential turnover that is responsible for the absence of meaningful results. Hence the poor conversion of tryptophan to indoleacetic acid and indolepyruvate may be construed to be caused by some other factor or factors. The most obvious of these is that the tryptophan in metabolised to products other than indoleacetic acid and indolepyruvate at such a rate that indolepyruvate formation is suppressed. These reactions could include tryptophan catabolism and alkaloid synthesis, and since these potentially use enzymes similar to those suspected of being involved in indoleacetic acid biosynthesis (Mazelis, 1980) it would be difficult to eliminate these, using inhibitors, from the incubation mixture.

Alternatively, the conversion to indolepyruvate is occurring, but it is breaking down too quickly to be utilised by the enzymes converting it to indoleacetic acid, and thus neither of the compounds can be detected. This is feasible since the only agent which could protect the indolepyruvate formed is the isoascorbate, which was added at the
extraction phase to prevent inactivation of the enzymes by quinones during extraction, and so may be depleted.

The breakdown of indolepyruvate leads to a large number of different compounds, including about 5% indoleacetic acid (Bentley et al., 1956). This makes it extremely difficult to determine indolepyruvate levels using estimation of the breakdown products, and indeed many of the products have properties similar to tryptophan making reproducible separation impossible. Thus, despite indolepyruvate being formed in the incubation, its breakdown would make the apparent conversion from tryptophan appear to be the same as in incubation of extracts with no enzymic activity.

A final possibility for the inconclusive results is that there is some problem with the enzymes in the pathway. This could be caused by a number of reasons: the cofactors supplied may not have been sufficient, or the enzymes may not have been completely extracted. If an enzyme were membrane bound, which is unlikely, then it would have been precipitated during the centrifugation step in the extraction. Or the enzyme may be highly labile and, despite the protection given by the isoascorbate and the polyvinylpolypyrrolidone, was degraded and unable to catalyse the conversion.

Also, with the enzymes, it is possible that in vivo they are concentrated within one cellular compartment, and so the intermediates do not have to diffuse very far to reach the next enzyme. However, in the extract, not only have the enzymes been diluted by the extraction buffer, but also by the presence of other enzymes, thus decreasing the probability of the intermediate reaching the enzyme. As well as decreasing the turnover of the enzymes, this would also make nonspecific breakdown of indolepyruvate more prevalent, because of
the greater time it spends in the extract not bound to enzymes and susceptible to attack by oxidants.

The inconclusive nature of the results are therefore probably caused by dilution of the enzymes involved in the formation of indoleacetic acid and the breakdown of the intermediates during incubation of the extracts. It may be possible to modify the experiment, by extracting in less buffer, or looking at specific organelles to decrease the dilution of the enzymes, and by adding antioxidants such as BHT to prevent indolepyruvate breakdown.
3. INDOLEACETIC ACID AND INDOLEPYRUVATE IN CELL SUSPENSION CULTURES

3.1. Introduction

The free indoleacetic acid content of plant tissue cultures has been shown to be correlated with growth of the culture (El-Bahr et al., 1984, 1987; Mousdale, 1985). These studies show that the indoleacetic acid concentration reaches a maximum when the growth rate is at the maximum level. There are some exceptions to this progression, where the indoleacetic acid levels remained at relatively low and constant levels throughout the growth of the cell cultures (Mousdale, 1982; Wyndaele et al., 1985).

This regular change in indoleacetic acid levels makes cell cultures an excellent tool in the study of the biosynthesis of the auxin. Suspension cultures are especially good, since the growth from subculture to the end of the exponential growth phase under controlled conditions is repeatable over long periods of time.

The role of indolepyruvate in the formation of indoleacetic acid can be studied by determining the relationship between the levels of both indoleacetic acid and indolepyruvate during the growth of the suspension culture. This relationship is determined by the amount and rate of the enzymes catalysing the synthesis of indoleacetic acid from indolepyruvate. The dependence of indoleacetic acid levels on indolepyruvate concentrations can follow several different patterns depending on whereabouts of the control of synthesis within the pathway.

One possible pattern is one in which the control of indoleacetic acid synthesis occurs after indolepyruvate formation. In this case the
conversion of indolepyruvate from the large tryptophan pool would be uncontrolled, producing indolepyruvate continually. The change in indolepyruvate concentration would be dependent on the difference between the rate of synthesis and the rate of loss from the indolepyruvate pool, by indoleacetic acid synthesis, degradation and other transformations of indolepyruvate. Thus indoleacetic acid concentrations would not be linked directly to the amount of indolepyruvate.

Alternatively, the major point in control of indoleacetic acid could be in the formation of indolepyruvate. A change in indoleacetic acid concentration might expect to be paralleled by a similar change in indolepyruvate concentration. The levels of indolepyruvate might also fluctuate in different ways depending on the rate of the remaining enzymes of indoleacetic acid biosynthesis and whether there was any fine tuning of the biosynthesis in the remaining steps.

In the presence of further control of indoleacetic acid biosynthesis, the indolepyruvate level would tend to decrease at times when the indoleacetic acid concentration was high, since more of the indolepyruvate is being converted than at times when the indoleacetic acid concentration is lower.

However, if the remaining steps in the formation of indoleacetic acid are only dependent on the rate of the enzymes involved - indolepyruvate decarboxylase and indoleacetaldehyde reductase or indoleacetaldehyde oxidase - then two different correspondences between the levels of indolepyruvate and indoleacetic acid are possible. The first of these is that if the rate of these enzymes are similar to the rate of the enzyme forming indolepyruvate, then changes in the concentration of indoleacetic acid will follow changes in the concentration of
indolepyruvate, i.e. if indolepyruvate increases to a high level, then shortly after indoleacetic acid concentration will increase.

The alternative to the above possibility is that if the rate of the enzymes is much faster than the rate of indolepyruvate formation, then the indolepyruvate concentration would remain fairly constant despite any changes in indoleacetic concentrations.

Several examples of varying changes in intermediate concentrations due to location of the control mechanism may be observed in the change in the levels of some of the GAs in spinach on the change from short day to long day conditions (Zeevaart et al., 1981; Gilmour et al., 1986). This change in photoperiod increased the concentration of GA$_{20}$ and GA$_{29}$, but decreased the concentration of GA$_{19}$. The GA$_{29}$ is synthesized from GA$_{19}$ via GA$_{20}$ (see Section 1.3.2) and the enzyme converting GA$_{19}$ to GA$_{20}$ has been shown to have almost no activity under short day conditions, and much more activity under long day conditions, while the conversion of GA$_{20}$ to GA$_{29}$ was uncontrolled.

Since there was no control of the conversion of GA$_{20}$, it would be expected that GA$_{20}$ levels would increase just prior to an increase in GA$_{29}$ levels. The decrease in GA$_{19}$ occurred because at the initiation of long day conditions there was no GA$_{44}$, the immediate precursor to GA$_{19}$, and so the loss of GA$_{19}$ due to formation of GA$_{20}$ was not balanced by synthesis of GA$_{19}$. However, after a short period the synthesis of GA$_{19}$ will occur and so the GA$_{19}$ levels will fluctuate with no direct correlation to changes in GA$_{29}$ concentrations.
3.2. Materials and Methods

3.2.1. Materials

Chemicals

With the exception of the chemicals given below, all the chemicals used in these experiments were purchased from Sigma Chemical Company at Analar grade.

Agar: finest bacterial grade from Davis gelatine (NZ) Ltd.
L-Amino acid oxidase: from *Crotalus durissus* from Boehringer Mannheim GmbH.
Dichlorodimethylsilane: from Pierce.
Hexane: ChromAR HPLC grade from Mallinckrodt.
[6-13C]Indoleacetic acid: from Cambridge Isotope Labs.
Methanol: ChromAR HPLC grade from Mallinckrodt.
N-methyl-N-nitroso-p-toluene sulphonamide: Analar grade from Serva.
DL-[methylene-14C]Tryptophan, 1.9GBq/mmol from Amersham International.
L-[5-2H]Tryptophan: from Cambridge Isotope Labs.
L-[5-3H]Tryptophan, 0.9TBq/mmol from Amersham International.
Plant Material

Mung beans (*Vigna radiata*) were purchased from Woolworths Ltd. They were soaked for 16 hours prior to sowing on water saturated vermiculite. The beans were grown in the dark at approximately $20^\circ$C.

Columns

GC column: SGE (0.25mm id x 12m) BP-1 column.

Normal Phase HPLC column: Waters (3.9mm id x 150mm) 5μm Resolve silica column.

Polymer Reverse Phase (PRP) HPLC column: Brownlee Polymer RP (4.6mm id x 100mm, 10μm particle size).

Reverse Phase HPLC column: Brownlee (4.6mm id x 100mm) ODS-MP Speri-5 column.

Silanisation of Glassware

All glassware that came in contact with indolepyruvate or indoleacetic acid was silanised to prevent losses by absorbance to the glass and chemical conversion. Silanisation was performed by first heating the glassware to $90^\circ$C for an hour to remove any moisture, then rinsing the glassware with a 3:1 mixture of toluene: dichlorodimethylsilane. The glassware was then washed with hexane followed by acetone and then heated to $90^\circ$C to remove any remaining reagent.
3.2.2. Cell Cultures

Initiation

Mung bean seeds were sterilised by soaking in 3% hypochlorite solution for twenty minutes, rinsed ten times with sterile distilled water and then soaked overnight in sterile distilled water. Selected beans were sown on agar in sterilised containers and grown in the light at 25°C for four days. The hypocotyls were excised and placed on solid media, consisting of B5 medium (see Appendix 1) supplemented with 2mg/mL 2,4-D, 0.1mg/mL kinetin and 0.8% agar. The callus cultures that formed were grown at 26°C with a 16 hour photoperiod.

After three passages of the callus cultures, approximately 10g of callus was broken up using a spatula and added to a 150mL conical flask containing 50mL of liquid B5 medium supplemented with 2mg/mL 2,4-D and 0.1mg/mL kinetin. These were grown with continual shaking at 26°C with a 16 hour photoperiod. After four weeks, 25mL portions of these suspension cultures were strained through nylon cloth, to remove large aggregates of cells, into 100mL of liquid B5 medium supplemented with 2mg/mL 2,4-D and 0.1mg/mL kinetin.

These suspension cultures were then subcultured in a similar fashion every 29 days and used as the source of experimental tissue used for determining the amount of indolepyruvate and indoleacetic acid in growing tissue.
Growth Curves

Growth curves were constructed by measuring the change in both the dry and wet weights of the cells in the suspension cultures as they developed. The wet weight was found by weighing a wet Whatman No. 1 filter paper, filtering 10mL of the suspension culture and then reweighing the filter paper plus cells. The wet weight was the difference between the two weights; for each value four readings were made, and then the weights averaged. The dry weight was determined in a similar manner, except that the filter paper and the cells and filter paper were dried at 65°C for at least eight hours prior to weighing.

3.2.3. Synthesis of Labelled Internal Standards

Synthesis of [3H]-Indolepyruvate

High specific activity indolepyruvate was synthesized from [3H]-L-tryptophan using the method of Cooney and Nonhebel (1989). 4x10^6DPM of [3H]-tryptophan was dissolved in 400μL of 0.25mM phosphate buffer, pH7.0. To this solution 0.025 units of L-amino acid oxidase in 25μL of buffer and 1 unit of catalase in 10μL of buffer were quickly added. All reactants were mixed and the reaction vial wrapped in aluminium foil to exclude light. The reaction was left at room temperature for ninety minutes and then stopped by the addition of 2mL of ice cold 5% citric acid solution.

The stopped reaction mixture was immediately applied to a Waters Sep-Pak C18 cartridge, which had been equilibrated with ice cold 5% citric acid. Unreacted tryptophan was eluted with 1.5mL of cold 20%
methanol in 1% aqueous acetic acid, and then the indolepyruvate eluted with 2mL cold methanol; 1mg of the antioxidant BHT was added to this fraction, which was then stored at -70°C for up to two days before use.

The yield of the reaction to indolepyruvate was determined at the same time as the indolepyruvate was being used so that any losses due to degradation during storage would not affect the calculations of amount of indolepyruvate. Actual [3H]-indolepyruvate in the final fraction was determined by comparing the specific radioactivity in the final fraction to the specific radioactivity in the indolepyruvate peak of an aliquot of the [3H]-indolepyruvate chromatographed with 1µg of unlabelled indolepyruvate. The method of chromatography used was polymer reverse phase HPLC as described in Section 2.2.3. The yields of the conversion varied between 10 and 20%.

**Synthesis of Deuterated Indolepyruvate**

Deuterated indolepyruvate was synthesized from [2H₅]-L-tryptophan using a modification of the previous method. 100µg (480nmol) of [2H]-tryptophan was dissolved in 400µL of 0.25mM phosphate buffer, pH7.0 in a small silanised glass vial. Approximately 180,000DPM of [3H]-tryptophan was also added as a tracer for the reaction; 0.3 units of L-amino acid oxidase and 1 unit of catalase were added in 25µL and 10µL of buffer respectively. The vial was capped, mixed thoroughly and then covered in aluminium foil to exclude light. The contents were left to react at room temperature for three hours.

The reaction was terminated by the addition of 770µL of methanol. The deuterated indolepyruvate was recovered using polymer reverse phase HPLC as described in Section 2.3.3, chromatographing the sample
in four 300µL aliquots to avoid overloading the column. The yield of the reaction was determined using the added radiotracer, and was only 2 to 2.5%. The methanol was removed from the deuterated indolepyruvate solution under a stream of nitrogen, then the remaining solvent removed by freezedrying. The indolepyruvate was stored dry with a dessicant at -70°C.

3.2.4. Extraction and Isolation of Indoleacetic Acid and Indolepyruvate

This method was based on the method used by Cooney and Nonhebel (1991b) to isolate tryptophan, indoleacetic acid and indolepyruvate from tomato shoots. All the glassware used in the extraction was silanised using the method given in Section 3.2.1 to prevent loss of the compounds by absorption to the glass surface and production of indoleacetic acid from tryptophan. Additionally, the initial steps of extraction and derivatisation of the indolepyruvate and indoleacetic acid were carried out in conditions of complete darkness or low light (natural light filtering through closed blinds) to prevent light mediated degradation of the compounds.

Harvesting Cells

Cells were harvested by centrifuging the suspension cultures at 10,000xg for five minutes, the resulting slurry precipitate was then filtered on Whatman No. 1 filter paper under aspiration. The resulting precipitate was weighed and then stored at -70°C until required.
Extraction

Approximately 10g of the cells were extracted at a time. The frozen cells were added directly to a flask containing 80mL of redistilled acetone, which had been made 1mM with respect to BHT and acidified with a few drops of glacial acetic acid. Internal standards of 100ng $^{2}$H-IPyA, 2 to 2.5kBq of $^{3}$H-IPyA and 1.2 to 1.7kBq of $^{3}$H-indoleacetic acid were added to the extraction. The flask was covered in aluminium foil to exclude light and kept on ice. The cells were sonicated using an MSE Soniprep 150 sonicator for five minute periods 5, 30 and 60 minutes after they were added to the acetone.

At the end of the third period of sonication, the extract was filtered using Whatman No. 1 filter paper, and then the acetone removed in vacuo, using a Buchi Rotavapor RE at 25°C, to the aqueous phase. This aqueous phase was refiltered, together with three 1mL washes of the evaporation flask using cold 1% acetic acid.

Seprolyte C$_{18}$

The Seprolyte C$_{18}$ column was equilibrated with at least two volumes of 1% acetic acid. The refiltered extract was applied to the column at approximately 5mL/min; then the column was washed with 2mL of cold methanol:1% acetic acid (30:70), the column was run dry at this stage. The indolepyruvate and indoleacetic acid were eluted using 3mL of cold methanol. A single crystal of BHT (approximately 1mg) was added to the eluent to prevent oxidation of the indolepyruvate. The
eluent was immediately used for the next step: the derivatisation of the indolepyruvate.

**Pentafluoroyl Benzyl Derivativisation of Indolepyruvate**

Approximately 0.15mL of a 20mg/mL solution of O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride in methanol was added to the eluent in a small silanised vial. The vial was flushed with nitrogen, capped and sealed with Parafilm. The reaction was left at room temperature for ten minutes, then reacted at 50°C for another fifty minutes. The excess reagent was reacted with 300μL of acetone, and the resulting solution was stored at -70°C for no longer than 24 hours.

**Separation of PFB-IPyA and IAA**

The products from the derivitisation of indolepyruvate were reduced *in vacuo* to approximately 0.5mL, and then diluted to 5mL with 1% acetic acid. This was applied to a Waters Associates C₁₈ Sep-Pak cartridge, which had previously been wetted with 5mL of methanol and then equilibrated with 10mL of 1% acetic acid. The cartridge was washed with 2mL of 1% acetic acid, then the indoleacetic acid fraction eluted with 3mL of methanol:1% acetic acid (60:40). The pentafluorobenzyl-indolepyruvate (PFB-IPyA) fraction was eluted with 2mL of methanol.

The methanol was removed from the indoleacetic acid fraction *in vacuo* and then the sample was freeze dried. The PFB-IPyA fraction was reduced to near dryness *in vacuo* and redissolved in 500μL of methanol:1% acetic acid (75:25) for reverse phase HPLC.
Reverse Phase HPLC of PFB-IPyA

The HPLC apparatus used for this and all subsequent HPLC steps consisted of Waters Model 501 and 510 pumps, controlled by a Waters Automated Gradient Controller. The detector used was a Waters Model 441 Absorbance Detector, set at 280nm.

Conditions for the reverse phase separation of PFB-IPyA were a solvent mixture of 75:25 of methanol:1% aqueous acetic acid at a flowrate of 0.5mL/min. The separation was achieved using isocratic conditions, with a retention time for PFB-IPyA of approximately 7.2 minutes. After each sample the column was washed with methanol for fifteen minutes to remove all of the applied sample, then equilibrated with the isocratic solvent for at least thirty minutes before applying the next sample.

The sample was collected into a small (1mL volume) vial. It was dried down under a stream of nitrogen, and then redissolved in 200μL of methanol for methylation.

Methylation of PFB-IPyA

Methylation of PFB-IPyA was carried out using a solution of diazomethane in ether (Schlenk and Gellerman, 1960). The diazomethane solution was formed from N-methyl-N-nitroso-p-toluene sulphonamide. Approximately 0.5g of N-methyl-N-nitroso-p-toluene sulphonamide was dissolved in 20mL of ether in a conical flask equipped with a sidearm. 1mL of absolute ethanol was added. The
reaction was initiated by the addition of approximately 2mL of saturated potassium hydroxide solution. The flask was warmed, using a waterbath, to approximately 40°C and the diazomethane distilled through the sidearm into a covered beaker in ice, containing approximately 10mL of ether. The diazomethane solution was prepared freshly each day it was required.

Methylation was performed by adding approximately 0.5mL of the diazomethane solution to the PFB-IPyA sample in methanol, and leaving to react at room temperature for thirty minutes. The sample was then reduced to dryness, redissolved in 200μL of isopropanol and stored at -70°C.

**Normal Phase HPLC of PFB-MeIPyA**

Conditions for the normal phase separation of the PFB-MeIPyA were a 90:10 hexane; 20% isopropanol in hexane solvent at 0.7mL/min. Separation was carried out isocratically in these conditions, i.e. 2% isopropanol in hexane. The retention time for the PFB-MeIPyA varied from 8.0 to 8.9 minutes, but was usually about 8.6 minutes.

The PFB-MeIPyA samples were collected into small vials, dried under a stream of nitrogen, redissolved in 200μL of isopropanol and stored at -70°C prior to quantification.

**Reverse Phase HPLC of IAA**

TEA solution: 1% aqueous acetic acid, adjusted to pH3.5 using triethylamine.
The freezedried IAA samples from the C18 Sep-Pak were dissolved in 200μL of 25:75 methanol:TEA solution. An aliquot of 5μL was taken and the specific radioactivity determined. This was used to calculate the proportion of IAA remaining in the sample at this stage. Stable labelled IAA internal standard was added for GC-MS SIM of IAA levels. The amount added was varied in such a way that it was equivalent to 100ng of the labelled compound being added at the extraction.

Samples were then analysed by reverse phase HPLC. The separation was carried using a gradient, of from 25% methanol:75% TEA solution to 100% methanol in ten minutes, and holding at pure methanol for a further ten minutes; the flowrate was 0.5mL/min. After each run the initial conditions were held for at least thirty minutes to re-equilibrate the column. The retention time for the IAA was 16.2 min. The methanol was removed from the samples in vacuo and the freezedried.

Formation the Pentafluoryl Benzyl Derivative of IAA

The freezedried IAA samples were dissolved in 1mL of methanol, transferred to a Reactivial and dried down under a stream of nitrogen. The sample was immediately redissolved in 200μL of 4mM tetraethyl ammonium hydroxide in methanol, then reduced to approximately 5μL under a stream of nitrogen. Next 100μL of dimethylacetamide was added followed by 15μL of pentafluorobenzyl bromide. The Reactivial was capped and vigorously mixed, then the reaction was allowed to proceed at room temperature for fifteen minutes, with constant stirring. The reaction was stopped by the addition of 100μL of water.
The pentafluorobenzyl-indoleacetic acid (PFB-IAA) was extracted from the reaction mixture by partitioning three times against 300μL of hexane. Following each addition of hexane the partitioning mixture was stirred vigorously for five minutes. The combined hexane phases were reduced to dryness under nitrogen, then the PFB-IAA redissolved in 100μL of isopropanol and stored at -70°C.

Normal Phase HPLC of PFB-IAA

Conditions for the normal phase separation of the PFB-IAA were identical to those used for the separation of PFB-IPyA: 2% isopropanol in hexane at a flowrate of 0.7mL/min. The retention time varied between 7.2 and 8.6 minutes, but was normally approximately 8.0 minutes.

The PFB-IAA samples were collected directly into small vials from the detector outlet, dried under a stream of nitrogen, redissolved in 200μL of isopropanol and stored at -70°C prior to quantification.

3.2.5. Quantification of Indoleacetic Acid and Indolepyruvate by GC-MS

The equipment used for the quantification of indolepyruvate and indoleacetic acid was a 12m, 0.25mm i.d. SGE BP-1 glass capillary column in a Hewlett Packard 5890 Series II gas chromatograph connected to a Hewlett Packard 5971A Mass Selective detector. Ion formation in the detector was carried out by electron impact, with a
70 eV ionising voltage, and the detector was run in selective ion monitoring (SIM) mode.

The temperature program for the gas chromatograph was:

- 0.5 min @ 150°C
- 20°C/min to 220°C
- 1 min @ 220°C
- 5°C/min to 240°C.

Under these conditions the retention time for indoleacetic acid was 5.45 minutes and the retention time for indolepyruvate was 7.45 minutes.

Both indoleacetic acid and indolepyruvate were monitored at the base peak, produced by the quinolinium ion (see Fig. 3.1), with an m/z ratio of 130 for the unlabelled compound, 135 for the deuterated indolepyruvate and 136 for the $^{13}$C indoleacetic acid. For approximately one third of the indoleacetic acid samples the ratio between unlabelled and labelled indoleacetic acid was also determined using the molecular ion (355:361); this ratio was found to be the same as the 130:136 ratio. This was not possible for the indolepyruvate samples or the remainder of the indoleacetic acid samples due to the small sample size. The ratio of the two peaks sizes was determined using the integrator for most samples, but in some cases where peaks were too small to be integrated, the cut and weigh method was employed.

The conversion of this ratio of labelled to unlabelled compound into concentration of the compound was carried out using a different method for each of indolepyruvate and indoleacetic acid. For indoleacetic acid a calibration curve was determined for a number of different known ratios of the labelled and unlabelled compounds. This calibration curve was used to determine the actual ratio of unlabelled to labelled indoleacetic acid from the ratio calculated from the GC-MS SIM results.
Fig. 3.1. The Quinolinium Ion.

This is the major fragment ion for both indoleacetic acid and indolepyruvate, with an m/z ratio of 130. $^2$H and $^{13}$C labels for the labelled indolepyruvate and indoleacetic acid were all within the indole ring of the structures, and so are all contained within the quinolinium ion. Thus, the fragments from the labelled indolepyruvate and indoleacetic acid had m/z ratios of 135 and 136 respectively.
The amount of indoleacetic acid in the sample was then calculated by multiplying the true ratio by the amount of labelled indoleacetic acid added to the sample. The labelled indoleacetic acid was added just prior to reverse phase HPLC, however the amount added to each sample was varied to take into account any losses up to that stage, so that each sample had the equivalent of 100ng of labelled indoleacetic acid added at extraction. The amount of indoleacetic acid per gram of fresh weight was determined by dividing the amount of indoleacetic acid by the weight of suspension culture extracted.

However, with indolepyruvate insufficient labelled indolepyruvate was synthesized to allow construction of a calibration curve. Instead, the ratio of 100ng unlabelled indolepyruvate to the amount of labelled indolepyruvate, approximately 100ng, was determined using GC-MS SIM. This standard ratio, 130*/135, was used to convert the observed sample ratio, 130s/135, into a quantifiable value by dividing the latter by the former, to yield the ratio 130s/130*. The amount of indolepyruvate in the sample was obtained by multiplying this ratio by the amount of unlabelled indolepyruvate in the standard ratio, 100ng. The amount of indoleacetic acid per gram of fresh weight was determined by dividing the amount of indolepyruvate by the weight of suspension culture extracted.

In both the cases of indoleacetic acid and indolepyruvate, when the ratio of labelled to unlabelled compound was one, then the ratio of the labelled peak (m/z 136 and 135) to the unlabelled peak (m/z 130) was approximately one.
3.3. Results and Discussion

The yields of the purification of the indolepyruvate and indoleacetic acid, up to the GC-MS step, from the suspension culture are given in Table 3.1. As can be seen from the table, the purification yields for indolepyruvate were at the low level of about 1 to 3 percent. These yields were at the low end of the yields obtained by Cooney (1989), who obtained yields of 2 to 17 percent, and were probably due to the condition of the HPLC columns used. The yields for the purification of indoleacetic acid ranged between one percent and fifteen percent, and were also within the yields obtained by Cooney (1989).

Examples of the chromatograms obtained for the GC-MS SIM of indolepyruvate and indoleacetic acid are given in Figures 3.2 and 3.3 respectively. These show the clear separation of the compounds and the relative amounts of the stable isotope labelled internal standard and the unlabelled endogenous compound.

The results of the quantification of the indolepyruvate and indoleacetic acid are given in Table 3.1; the amounts of samples extracted and ratios of endogenous compound to internal tracer for each of the samples are given in Table 3.2 for indolepyruvate and Table 3.3 for indoleacetic acid and the calibration curve used to determine the amount of endogenous indoleacetic acid is given in Figure 3.4.

Indolepyruvate quantification was not possible in some cases since neither sample nor standard was detected. This is probably due to losses during purification, and the calculated yield was due to a breakdown
Fig. 3.2. GC-MS SIM Trace for Indolepyruvate.

The equipment and conditions for separation were as given in Section 3.2.5. The temperature program for the gas chromatograph was:

<table>
<thead>
<tr>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5min</td>
<td>@ 150°C</td>
</tr>
<tr>
<td>20°C/min to</td>
<td>220°C</td>
</tr>
<tr>
<td>1min</td>
<td>@ 220°C</td>
</tr>
<tr>
<td>5°C/min to</td>
<td>240°C</td>
</tr>
</tbody>
</table>

The retention time for indolepyruvate was 7.45 minutes.

These traces were produced from the first of the 29 day samples. They show the abundance of the 130.1 and 135.1 ions detected by the GC-MS SIM, peak areas are given in Table 3.2.
**Abundance**

**Ion 130.10: IPYA10.D**

![Graph of Ion 130.10: IPYA10.D]

- **Time ->**
  - 4.00
  - 5.00
  - 6.00
  - 7.00
  - 8.00
  - 9.00
  - 10.00

- **Abundance**
  - 2500
  - 2000
  - 1500
  - 1000
  - 500

- Peaks at:
  - 5.46
  - 7.46

**Abundance**

**Ion 135.10: IPYA10.D**

![Graph of Ion 135.10: IPYA10.D]

- **Time ->**
  - 4.00
  - 5.00
  - 6.00
  - 7.00
  - 8.00
  - 9.00
  - 10.00

- **Abundance**
  - 2500
  - 2000
  - 1500
  - 1000
  - 500

- Peaks at:
  - 3.67
  - 7.44
Fig. 3.3. GC-MS SIM Trace for Indoleacetic Acid.

The equipment and conditions for separation were as given in Section 3.2.5. The temperature program for the gas chromatograph was:

- 0.5min @ 150°C
- 20°C/min to 220°C
- 1min @ 220°C
- 5°C/min to 240°C.

The retention time for indoleacetic acid was 5.45 minutes.

These traces were produced from the second five day sample. They show the abundance of the 130.1 and 136.1 ions and also the abundance of the molecular ions at m/z 355 and 361. Peak areas for the quinolinium ions are given in Table 3.2; the ratio for the peak areas of the molecular ions was 0.52, compared with a ratio of 0.53 for the quinolinium ion.
This calibration curve was constructed by mixing together $^{13}$C-labelled and unlabelled indoleacetic acid in proportions ranging from 5:1 to 1:5 and then analysing the indoleacetic acid sample for the ratio of the peaks detected at m/z 130 and 136. The detection and quantification of the peaks was as given in Section 3.2.5. The curve drawn through the points was calculated by nonlinear regression.
MIZ 130:136 Measured by GC-MS SIM

Ratio of Unlabelled to Labelled Indoleacetic Acid

5.00 4.50 4.00 3.50 3.00 2.50 2.00 1.50 1.00 0.50 0.00

Parameter values on the x-axis and y-axis are not clearly visible due to the image's quality.
<table>
<thead>
<tr>
<th>Time (days)</th>
<th><strong>Indolepyruvate</strong></th>
<th></th>
<th><strong>Indoleacetic Acid</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yield (%)</td>
<td>Concentration (ng/g fwt)</td>
<td>Yield (%)</td>
<td>Concentration (ng/g fwt)</td>
</tr>
<tr>
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<td>0.5</td>
<td>-c</td>
<td>1.2</td>
<td>-c</td>
</tr>
<tr>
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<td>3.4f</td>
<td>2.9</td>
<td>19.4</td>
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<td>-c</td>
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<td>8.5</td>
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<td>-d</td>
<td>5.0</td>
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</tr>
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<td>11.0</td>
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</tr>
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<td>-a</td>
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<td>12.0</td>
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<td>-c</td>
<td>-b</td>
<td></td>
</tr>
<tr>
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<td>2.3</td>
<td>11.0</td>
<td>1.0</td>
<td>13.3</td>
</tr>
<tr>
<td>29</td>
<td>1.0</td>
<td>10.0</td>
<td>2.0</td>
<td>13.8</td>
</tr>
<tr>
<td>29</td>
<td>-a</td>
<td></td>
<td>11.0</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Table 3.1. Yields and Amounts of Indolepyruvate and Indoleacetic Acid From Mung Bean Suspension Cultures.

- = not determined
a = no deuterated indolepyruvate standard added.
b = sample lost during purification.
c = no internal standard detected by GC-MS SIM.
d = no sample detected by GC-MS SIM.
e = Sample contaminated.
f = Cut and weigh method used to determine concentration
product of the added radioactive tracer. In some cases the indolepyruvate peak for the sample was detected, but not that of the added internal standard. In these cases there was no sign at all of the labelled indolepyruvate, thus the lack of standard is either due to the amount of indolepyruvate in the sample being so great that the standard peak cannot be seen or, more likely the internal standard was not added to the extraction buffer during extraction, since this was performed in complete darkness.

With the quantification of indoleacetic acid these problems did not occur. However, there was a problem of contamination of the syringe used for the injection of the samples with the standard indoleacetic acid used for constructing the calibration curve. This contamination was possibly transferred to the vials containing two of the samples, and so the results of those samples were not used.

The concentrations of indolepyruvate and indoleacetic acid determined by GC-MS SIM are given in Table 3.1. The concentrations obtained for indoleacetic acid, which were between 2.5 and 26.5 ng/g fwt, are similar to those of vegetative tissue (see Table 1.1). The concentrations determined are also similar to the approximately 4 ng/g fwt indoleacetic acid determined for soybean callus cultures (Wyndaele et al., 1985) and tobacco suspension cultures (Mousdale, 1982).

The concentrations of indolepyruvate were between 3.4 and 12.1 ng/g fwt. These values are close to the concentration of 5.7 ng/g fwt determined for tomato shoots (Cooney and Nonhebel, 1991b); the only published determination of indolepyruvate concentration in plants.

The change in the concentrations of indoleacetic acid and indolepyruvate with the development of the suspension culture are given in Figure 3.5. As can be seen in the figure, there is little obvious
<table>
<thead>
<tr>
<th>Time Days</th>
<th>Sample Weight g</th>
<th>Peak Area 130</th>
<th>Peak Area 135</th>
<th>130:135 Ratio</th>
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</thead>
<tbody>
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<td>9.5</td>
<td>125833</td>
<td>-</td>
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Table 3.2. Sample Weights and Peak Areas for Indolepyruvate Analysis by GC-MS SIM.

- = no peak detected.

<sup>a</sup> = peak ratio determined by cut and weigh method.
<table>
<thead>
<tr>
<th>Time</th>
<th>Sample weight (g)</th>
<th>Peak Area</th>
<th>130:136 Ratio</th>
</tr>
</thead>
<tbody>
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<td>Days</td>
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<td>90397</td>
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</tr>
<tr>
<td>29</td>
<td>9.2</td>
<td>72224</td>
<td>177278</td>
</tr>
</tbody>
</table>

Table 3.3. Sample Weights and Peak Areas for Indoleacetic Acid by GC-MS SIM.
Fig. 3.5. Indoleacetic Acid and Indolepyruvate Concentrations Throughout Development of Mung Bean Suspension Culture.

Concentrations of indoleacetic acid (■) and indolepyruvate (○) were determined by GC-MS SIM as described in Sections 3.2.4 and 3.2.5. Each point is the value of a single determination. The time axis is the number of days after subculture that the sample of suspension culture was taken for analysis.
change in the indoleacetic acid concentrations with growth of the culture with the concentration staying at around 7 ng/g fwt; although the earlier concentrations of indoleacetic acid, those less than eight days, may well be higher, with the lack of samples during this time period hiding the fact. However the almost constant low level of indoleacetic acid parallels the results of Mousdale (1982) and Wyndaele and coworkers (1985) who also found almost unchanged indoleacetic acid concentrations with development of cultures.

In contrast indolepyruvate levels do seem to change during the growth of the suspension cultures: concentrations start at a low value and gradually increase to a maximum value, which is maintained for the remainder of the culture period. This relationship becomes even more significant when the concentrations are compared with the growth rate of the culture (Figure 3.6): the figure shows that the indolepyruvate levels reach the plateau concentration at about the same time as the cultures reach the log phase of growth. This suggests that the growth of the culture is correlated with the concentration of indolepyruvate.

Despite this apparent correlation between indolepyruvate concentration and growth, there is no obvious correspondence between indolepyruvate and indoleacetic acid concentrations. However, both compounds are maintained at similar low concentrations. This suggests that concentration of indolepyruvate is controlled and that control of the formation indolepyruvate is an important factor in the control of indoleacetic acid biosynthesis.

The lack of relationship between indolepyruvate and indoleacetic acid concentrations suggests that there are further points of control beyond the formation of indolepyruvate; yet the correspondence between indolepyruvate and growth rate implies the opposite. It is likely that there is some correlation between indolepyruvate and
Concentrations of indolepyruvate (○) were determined as described in Sections 3.2.4 and 3.2.5; each point is the value of a single determination. The continuous line is the growth rate of the cultures, this was determined using points from the wet and dry growth rates over three subcultures of the suspension cultures. The wet and dry weights were determined as described in Section 3.2.3, and the growth rate calculated as the natural logarithm of the weight divided by the initial weight of the culture (ln[W/W₀]).
indoleacetic acid concentrations, but that this correlation cannot be seen due to the scattered nature of the results obtained, and the experiment should be repeated; this was not possible due to time constraints.
4. PURIFICATION OF TRYPTOPHAN AMINOTRANSFERASE

4.1. Introduction

Aminotransferases are well known for having low substrate specificity, probably due to it being necessary for the same active site to bind with two different substrates one after the other. This makes it difficult to apply the E.C. numbering scheme to aminotransferases, and so trivial names are usually applied to them (Braunstein, 1973, Jenkins, 1985). As well as causing difficulty in naming, the low specificity hinders purification. There are many examples of enzymes being purified using an assay for one substrate that are found to be identical to another enzyme purified using a different substrate (Noguchi et al., 1979, Okuno et al., 1975). The low specificity also makes it difficult to determine whether an enzyme is pure or a mixture of two or more aminotransferases.

Tryptophan aminotransferases have suffered from these problems: the tryptophan aminotransferase found in rat brain was shown to be the cytosolic aspartate aminotransferase (Harada et al., 1978), and in the rat liver the tryptophan aminotransferase was found to be identical to to phenylalanine aminotransferase and tyrosine aminotransferase (Jacoby and LaDu, 1964). Indeed this latter phenomenon of combined specificity for all the aromatic amino acids is common, and tryptophan aminotransferases are usually found to be an aromatic aminotransferase.
As well as transaminating the aromatic amino acids, aromatic aminotransferases are usually able to transaminate aspartate, frequently at a much greater rate than tryptophan (Givan, 1980). This may be due to mixtures of aminotransferases being considered a single enzyme; however, the *E. coli* tyrosine aminotransferase has been isolated by mutation (Gelfand and Steinberg, 1977, Gelfand and Rudo, 1977) and has equal maximum rates utilising either tryptophan or aspartate.

The first investigation of a plant aromatic aminotransferase was carried out by Gamborg and Wettar (1963), who purified seventeenfold a mung bean enzyme capable of transaminating phenylalanine, tyrosine and tryptophan, although other amino acids were not tested as substrates. Truelson (1972) further purified the mung bean enzyme, obtaining an aminotransferase with the specificity shown in Table 4.1.

Wightman and Forest (1972b) purified a multispecific aminotransferase six hundredfold from bush bean (*Phaseolus vulgaris*) roots, demonstrating purity by a single band in gel electrophoresis. This was able to transaminate five amino acids: aspartate, glutamate, tryptophan, phenylalanine and tyrosine as shown in Table 4.1. As the table displays, the activity with aspartate was far higher than with the aromatic amino acids.

Other putative aromatic aminotransferases have also been purified. A tryptophan:glyoxylate aminotransferase was localised in the peroxisomes of spinach leaves (Noguchi and Hayashi, 1980), although its high activity with serine (Table 4.1) and lack of activity with 2-oxoglutarate, oxalacetate and pyruvate combined with its peroxisomal location suggest that this is a serine:glyoxylate aminotransferase involved in serine and glycine synthesis.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Relative Activity</th>
<th>Activity</th>
<th>Trp = 100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mung Bean(^a)</td>
<td>Bush Bean(^b)</td>
<td>Spinach(^c)</td>
</tr>
<tr>
<td>L-glycine</td>
<td>0</td>
<td>0</td>
<td>821</td>
</tr>
<tr>
<td>L-alanine</td>
<td>202</td>
<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>L-valine</td>
<td>62</td>
<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>L-leucine</td>
<td>248</td>
<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td>0</td>
<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>4</td>
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<td>&lt;10</td>
</tr>
<tr>
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<td>216</td>
<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>L-serine</td>
<td>0</td>
<td>0</td>
<td>1530</td>
</tr>
<tr>
<td>L-threonine</td>
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<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>L-arginine</td>
<td>213</td>
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<td>&lt;10</td>
</tr>
<tr>
<td>L-lysine</td>
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<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>L-aspartic acid</td>
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<td>1660</td>
<td>&lt;10</td>
</tr>
<tr>
<td>L-proline</td>
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<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>L-phenylalanine</td>
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<td>158</td>
<td>&lt;10</td>
</tr>
<tr>
<td>L-tyrosine</td>
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<td>114</td>
<td>&lt;10</td>
</tr>
<tr>
<td>L-histidine</td>
<td>82</td>
<td>33</td>
<td>&lt;10</td>
</tr>
<tr>
<td>D-tryptophan</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-phenylalanine</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-tyrosine</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-histidine</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1. Relative Specificities of Plant Aromatic Aminotransferases.

References

a: Truelson, 1972.
b: Forest and Wightman, 1972b.
c: Noguchi and Hayashi, 1980.
De-Eknamkul and Ellis (1987) purified three tyrosine aminotransferases from cell cultures of *Anchusa officinalis*, which are used for the biosynthesis of the alkaloid rosamarinic acid. Although neither rates nor Kms were given, these had varying specificities, which suggested that one was an aspartate aminotransferase with tyrosine aminotransferase activity, a second was involved in rosmarinic acid biosynthesis and the third was part of the pathway for synthesis of tyrosine or plastoquinone.

The use of affinity chromatography for the quick efficient purification of plant aminotransferases, as used for animal ones, is not possible since the pyridoxal phosphate group, used for the affinity basis, is more tightly held within the enzyme. Instead a large variety of methods are used for purification. Stepwise ammonium sulphate precipitation is used for crude separation of different types of aminotransferases; for example 0-60% saturation is used for purification of carrot aspartate aminotransferase (Turano et al., 1991), while 60-80% saturation is used for mung bean tryptophan aminotransferase (Truelson, 1972). Gel filtration is frequently used (Kutacek, 1985) and so is ion exchange chromatography, usually with DEAE (Gamborg, 1963). However, there are no standard methods of purification and the varying characteristics of the many aminotransferases means that purification of them must be approached empirically.
4.2. Materials and Methods

4.2.1. Materials

Chemicals

With the exception of the chemicals given below, all the chemicals used in these experiments were purchased from Sigma Chemical Company at Analar grade.

Acetic acid: AnalaR grade from BDH.
Acrylamide: electrophoresis purity from BioRad.
Ammonium persulphate: Electran grade from BDH.
Ammonium sulphate: HPLC grade from Bio-Rad.
Hydrochloric acid: AR grade from Mallinckrodt.
L-[5-3H]Tryptophan, 0.9TBq/mmol from Amersham International
Methanol: AnalaR grade from BDH.
Nitric acid: AR grade from JT Baker.
Potassium and dipotassium phosphate; GPR grade from BDH.
Sodium chloride: crystalline research grade from Serva.
Sodium dodecyl sulphate: electrophoresis purity from BioRad.
TEMED: Electran grade from BDH.
Plant Material

Mung beans (*Vigna radiata*) were purchased from Woolworths Ltd. They were soaked for 16 hours prior to sowing on water saturated vermiculite.

Columns

The following columns and column packing materials, purchased from Pharmacia LKB were used:

- **Sephacryl S-300HR**: 80cm x 2.5cm diameter column packed using Sephacryl S-300 High Resolution beads.
- **Fastflow Q**: 8cm by 1cm diameter column packed using Q-Sepharose Fastflow beads.
- **Mono Q**: 50mm by 5mm diameter Mono Q HR 5/5 column.
- **Phenylsuperose**: 50mm by 5mm diameter Phenyl Superose HR 5/5 column.
- **Superose-12**: 300mm by 10mm diameter Superose-12 HR 10/30 column.
4.2.2. Assays

Tryptophan Aminotransferase Assays

Spectrophotometric Assay

Assay buffer: 0.1M borax, 0.5mM Na$_2$HAsO$_4$, 0.5mM EDTA, adjusted to pH8.5 with glacial acetic acid.

Tryptophan solution: 0.013M tryptophan in assay buffer.

2-Oxoglutarate solution: 0.13M 2-oxoglutarate in assay buffer.

Pyridoxal phosphate solution: 0.6mM pyridoxal phosphate in assay buffer.

This assay was based on the method of Lin and coworkers (1951), and involved the stabilization of the indole pyruvate formed as an enol complex with borax, catalysed by the arsenate.

The assay was performed by mixing 1.0mL of tryptophan solution, 0.1mL of pyridoxal phosphate solution and 0.1mL of suitably diluted enzyme extract. This was pre-incubated at 37°C for five to ten minutes, then the assay was started by adding 0.1mL of the 2-oxoglutarate solution, and then incubated at 37°C for one to four hours. The reaction was then stopped by adding 0.1mL of 70% perchloric acid and the absorbance of the borate-enol complex was measured at 328nm against a blank sample treated in the same way, i.e. 0.1mL of assay buffer instead of enzyme extract. In cases when the extract was coloured, two sets of assays were set up: one was measured at zero time and the other after incubation, to allow for the interference by the extract. The extinction coefficient for the indolepyruvate borate complex was the value determined by Truelson (1972) of 9800 Lmol$^{-1}$cm$^{-1}$. The dilution
of the enzyme extract and the time of the incubation were chosen, by estimation, to minimize the amount of extract used, the effects of coloured extracts and to give an absorbance of 0.2 to 0.4 after the incubation.

Radioassay

Assay buffer: 0.1M borax, 0.5mM Na₂HAsO₄, 0.5mM EDTA, 1.3mM pyridoxal phosphate, pH 8.5.

Substrate solution: 7mM tryptophan, 35mM 2-oxoglutarate in assay buffer.

Radiolabel: 10⁶DPM/mL 5-[³H]-L-tryptophan.

Cold trap: 1mg/ml indolepyruvate, 1mM butylated hydroxytoluene in methanol.

This method was a modification of the method given in Section 2.2.4, although exact the quantification involving the use of the HPLC was not used.

The assay was carried out by preincubating 2.5mL of enzyme extract with 2.5mL of assay buffer for five minutes at 37°C, then adding 0.1mL of the radiolabel. The reaction was initiated by adding 0.1mL of the substrate solution and the mixture incubated at 37°C for 45 minutes.

The reaction was stopped by adding 0.1mL of glacial acetic acid and 0.1mL of cold trap, and then immediately applied to the Sepralyte C18 column, which had been equilibrated with pH 2.5 aqueous acetic acid. The column was washed with 5mL of the aqueous acetic acid and then the tryptophan eluted with 10mL of 40% methanol in aqueous acetic acid. The indolepyruvate was eluted using 100% methanol. Finally
200μL samples of all fractions were counted as in Section 2.2.4 and the conversion to indolepyruvate determined.

Tyrosine Aminotransferase Assay

Assay buffer: 0.2M potassium phosphate, pH7.4.
Tyrosine solution: 6.85mM in assay buffer. A supersaturated solution prepared by heating to 90°C to dissolve, then cooling to 37°C and using straight away.
Pyridoxal phosphate solution: 1.3mM in assay buffer.
2-Oxoglutarate solution: 0.3M in assay buffer.

This assay was used in the initial steps of purification on the assumption that the enzyme being purified had an aromatic specificity and, since hydroxyphenylpyruvate is much more stable than indolepyruvate, this would give a good indication of which fractions the aromatic aminotransferase activity was present. In all cases the results were verified using one of the tryptophan assays given above. The assay method used was the Diamondstone Method (Diamondstone, 1967).

The assay was carried out by preincubating 2.8mL of the tyrosine solution, 0.1mL of the pyridoxal phosphate solution and 0.2mL of the enzyme extract at 37°C for ten minutes. Then 0.1mL of the 2-oxoglutarate solution was added to start the reaction, and the mixture incubated at 37°C for one to two hours. The incubation was stopped by adding 0.1mL of a 40% sodium hydroxide solution, and left to stand at room temperature for thirty minutes to allow conversion of the hydroxyphenylpyruvate to hydroxybenzaldehyde. The absorbance of
this was measured at 331nm against a blank treated in the same method. In most cases a zero time reading was made in the same manner as in the tryptophan aminotransferase assay and subtracted from the absorbance after the incubation to allow for coloured extracts. The extinction coefficient used for quantification of the results was 19900Lmol⁻¹cm⁻¹.

Aspartate Aminotransferase Assay

Assay buffer: 0.2M potassium phosphate, pH7.4.
Aspartate solution: 0.2M aspartate in assay buffer.
NADH solution: 1mg/mL in assay buffer.
Malic dehydrogenase: 700U/mL in assay buffer.
2-Oxoglutarate solution: 0.1M 2-oxoglutarate in assay buffer.

This assay was used to determine the activity of what was considered to be the major contaminant aminotransferase activity: the glutamate:oxalacetate aminotransferase involved in transporting reducing power to chloroplasts and in transporting carbon dioxide. The assay used was a modification of the method of Karmen and co-workers (1953).

The assay was carried out by mixing 0.5mL of aspartate solution, 0.2mL of NADH solution, 0.1mL of malic dehydrogenase and up to 2.0mL of enzyme extract. the volume was made up to 2.8mL using assay buffer. Initially, the absorbance of the mixture was monitored at 340nm to see if there were any components in the enzyme extract capable of oxidising the NADH; however, none were detected, even in crude extracts, so this was not carried out in later assays.
Then 0.2mL of the 2-oxoglutarate solution was added and the absorbance at 340nm was monitored constantly for ten to twenty minutes against an air blank. A control was performed using no enzyme extract and subtracted from the enzyme assays. The extinction coefficient used for NADH was 6220Lmol⁻¹cm⁻¹.

4.2.3. Protein Estimation

Protein estimation was carried out using Bradford's method (1976). The Bradford reagent was made by dissolving 50mg of Coomassie brilliant blue G-250 in 25mL of ethanol and then adding 50mL of 80% orthophosphoric acid. This was diluted to a final volume of 500mL by adding water slowly. the resulting solution was filtered using Whatman No. 1 filter paper, until it was no longer blue. The reagent was stored at 4°C, and periodically refiltered to remove any precipitate that formed.

Measurements of protein content were made by adding 2.5mL of reagent to 0.05mL of sample, containing 5-50μg of protein, and immediately mixing thoroughly. The absorbance was read at 595nm between two and sixty minutes after mixing. The protein concentration was determined using a BSA calibration curve prepared and measured at the same time as the sample.
4.2.4. Gel Electrophoresis

Monomer solution (30% T, 2.7% C): 58.4g acrylamide, 1.6g Bis, add water to 200mL and filter. Stored at 4°C in the dark.

4 x Running gel buffer: pH8.8 1.5M Tris-HCl.
4 x Stacking gel buffer: pH6.8 0.5M Tris-HCl.

Running buffer: 12g Tris, 57.6g glycine, 40ml 10% SDS in water, made up to 4L with water.

2 x Loading solution: 2.5mL 4 x stacking gel buffer, 4.0mL 10% SDS, 2.0mL glycerol, 1mL 2-mercaptoethanol, 0.1mL 0.2% bromothymol blue solution, make up to 10mL with water, divide into 10μL aliquots and freeze.

Stain stock: 1% Coomassie blue R-250.
Stain solution: 0.125% Coomassie blue R-250, 50% methanol, 10% acetic acid; made up of 62.5mL stain stock, 250mL methanol, 50mL glacial acetic acid, up to 500mL with water.

Destaining solution 1: 50% methanol, 10% acetic acid.
Destaining solution 2: 7% acetic acid, 5% methanol.
Desalting buffer: 50mM NH₄HCO₃, 0.02% SDS.

The method of gel electrophoresis used was the sodium dodecylsulphate polyacrylamide technique of Laemmli (1970). A 10% acrylamide concentration was used in all gels, and the amounts of each of the solutions in the stacking and separating gels is given in Table 4.2. Gels were poured three at a time and stored wrapped in polyethylene film at 4°C for not more than a week. Samples were dialysed against desalting buffer to remove any salts and then freeze dried. The sample was then redissolved in 5 to 10μL of 2 x loading buffer and diluted with
an equal volume of water. The sample was boiled for approximately two minutes prior to being loaded onto the gel.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Separating gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% T 2.7% C</td>
<td>20 mL</td>
<td>2.66 mL</td>
</tr>
<tr>
<td>4 x Running gel buffer</td>
<td>15 mL</td>
<td>-</td>
</tr>
<tr>
<td>4 x Stacking gel buffer</td>
<td>-</td>
<td>5.0 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.6 mL</td>
<td>0.2 mL</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>24.1 mL</td>
<td>12.2 mL</td>
</tr>
<tr>
<td>10% Ammonium persulphate</td>
<td>0.3 mL</td>
<td>0.1 mL</td>
</tr>
<tr>
<td>TEMED</td>
<td>20 mL</td>
<td>10 mL</td>
</tr>
</tbody>
</table>

Table 4.2. Recipe for 10% Polyacrylamide Gel.

The separation was effected at a constant voltage of 100mV until the bromothynol blue dye front reached the bottom of the gel. Gels were stained for approximately an hour, and then destained to the desired level. In some cases insufficient protein had been loaded to be visualised by Coomassie blue staining, and silver staining (Merrill et al., 1981), as described below, was used.

4.2.5. Silver Staining

40% Fixative: 40% ethanol, 10% acetic acid in water.
10% Fixative: 10% ethanol, 5% acetic acid in water.
Oxidiser: 2g \( \text{K}_2\text{Cr}_2\text{O}_7 \), 0.44mL 6.5% nitric acid made up to 200mL with water and stored at 4°C.

Silver reagent: 4.08g \( \text{AgNO}_3 \) dissolved in 200mL water and stored in a dark bottle at 4°C.

Developer: 0.28M \( \text{Na}_2\text{CO}_3 \). Just prior to use 0.1mL formaldehyde was added per 200mL of solution.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume mL</th>
<th>Time min</th>
</tr>
</thead>
<tbody>
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<td>30</td>
</tr>
<tr>
<td>10% Fixative</td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>10% Fixative</td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>Oxidiser</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>MilliQ Water</td>
<td>100</td>
<td>5</td>
</tr>
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<td>5</td>
</tr>
<tr>
<td>MilliQ Water</td>
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<tr>
<td>Silver reagent</td>
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<td>5</td>
</tr>
<tr>
<td>MilliQ Water</td>
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<td>0.5</td>
</tr>
<tr>
<td>Developer</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>Developer</td>
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<td>MilliQ Water</td>
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<td>30</td>
</tr>
<tr>
<td>MilliQ Water</td>
<td>100</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 4.3. Silver Staining Protocol.
The oxidiser and silver reagent were diluted tenfold prior to used and then heated to 30°C. The sequence of staining used is given in Table 4.3. The amount of time used for the developer varied depending on the amount of protein present.

4.2.6. Purification

Extraction buffer: 50mM K$_2$HPO$_4$, 0.5mM EDTA, 0.5mM MnCl$_2$, pH 8.5; made 10mM with respect to isoascorbate just prior to extraction. Gel filtration buffer: 0.1M NaCl, 10mm Tris-HCl, pH 8.0. Fastflow Q equilibration buffer: 10mM Tris-HCl, pH 9.0. Mono Q buffer A: 10mM Tris-HCl, pH 8.0. Mono Q buffer B: 1.0M NaCl, 10mM Tris-HCl, pH 8.0. Phenylsuperose buffer A: 1.7M ammonium sulphate, 50mm phosphate, pH 7.7. Phenylsuperose buffer B: 50mM phosphate, pH 7.7.

Preparation of Enzyme Extract

Mung beans were grown in the dark, on moist vermiculite, at a temperature of 20°C for six days. The primary leaves and the shoot from 2cm below the cotyledons to the apex, without the cotyledons, were harvested onto ice and extracted no more than an hour after harvesting.

Approximately 200g of plant material was extracted in a 1:1 ratio of plant material to extraction buffer, with a 1:0.05 ratio of plant
material to polyvinylpolypyrrolidone. The material was extracted in an Atomix blender for one minute at full speed, then for five minutes at half speed. The resulting homogenate was expressed through eight layers of cheese cloth. The residue was briefly extracted with another 50mL of extraction buffer, and filtered through the cheesecloth. The filtrate was centrifuged at 20,000xg for twenty minutes at 4°C, and the resulting filtrate was designated the crude extract.

**Ammonium Sulphate Fractionation.**

The ammonium sulphate fractionation used in the purification was the same protocol as that determined by Truelson (1972). Initially the extract was taken to 50% saturation, and the precipitated protein removed, then the extract was taken to 60% saturation and the precipitated protein removed. Finally the extract was taken to 80% saturation and the precipitated protein, which contained the aminotransferase was retained. This two step fractionation method was used since the aminotransferase would precipitate in the lower saturation solution if the concentration was taken directly to 60%.

Fractionation was carried out at 4°C at all times. At each step of increasing saturation the ammonium sulphate was added in solid form over a period of ten to twenty minutes, at a rate such that levels of undissolved ammonium sulphate were minimised. The extract was left to mix slowly for 45 minutes at each ammonium saturation level. Then the precipitated protein was removed by centrifuging the extract at 10,000xg for 15 minutes and the supernatant decanted off.
Gel Filtration

The 60-80% ammonium sulphate pellet was redissolved in about 10mL of gel filtration buffer. The concentrated enzyme solution was applied to the Sephacryl 300HR column at a flowrate of approximately 20mL/hour, and then eluted at a flowrate of 45mL/hour and collected in 6mL fractions using the same buffer.

Gel filtration using a Sepharose-12 column FPLC was also used later in the separation to check the molecular weight. The FPLC equipment used was all supplied by Pharmacia LKB, and consisted of two P-500 pumps a GP-250 gradient programmer, and a UV single path absorbance monitor; when the sample volume was greater than 300μL the sample was loaded using a Superloop™. This equipment was also used for the FPLC steps of Mono Q anion exchange and Phenylsuperose chromatography. The Superose-12 column was equilibrated with Mono Q buffer A, and was loaded with 100μL of the fraction from the Mono Q column. Sample loading and elution was performed at a flowrate of 0.4mL/min. In addition to the enzyme sample, a standard mix of 0.25mg catalase (MW 232kDa), 0.1mg β-galactosidase (MW 106kDa), 0.25mg BSA (MW 66kDa) and 0.3mg lysozyme (MW 14.1kDa) was chromatographed as a separate run to calibrate the molecular weight separation of the column.

Anion Exchange Chromatography

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The enzyme fraction from the S-300HR column was adjusted to pH 9.0 using 1.0M potassium hydroxide. It was applied to the Fastflow Q column, which had been equilibrated with Fastflow Q buffer. The column was then washed with the same buffer made 0.15M with sodium chloride. The column was eluted with a linear gradient of 0.15M to 0.45M sodium chloride in Fastflow Q buffer over a volume of 80mL, at a flowrate of approximately 30mL/hour, and 2.5mL fractions of eluent were collected.

The tryptophan aminotransferase activity peaks from three extractions were combined, diluted with an equal volume of Mono Q buffer A and spun at 10,000xg for ten minutes to remove any particulate material. The resulting supernatant was then applied to the Mono Q column, which had been equilibrated with a mixture of 12% buffer B in buffer A, at a flowrate of 1mL/min. After loading the sample, the column was washed for a few minutes, the flowrate was decreased to 0.25mL/min and then the sample eluted using a gradient of 12 to 20% buffer B in 24 minutes, then 20 to 25% B in another 36 minutes.

**Hydrophobic Interaction Chromatography**

The enzyme-containing fractions from the Mono Q separation (approximately 2.5mL ) were combined and dialysed against 1.5 L of Phenylsuperose buffer B for five hours. Frequently samples from more than one Mono Q column run were combined to minimise losses at this step. The dialysate was then made 1.7M with solid ammonium sulphate and spun at 13,000xg for ten minutes to remove any particulate material. The sample was applied to the Phenylsuperose column, which
had been equilibrated with Phenylsuperose buffer A, at a flowrate of 0.25mL/min. The extract was then eluted using a three step gradient: 0 to 40% buffer B in 15 minutes, then 40 to 60% buffer B in another 45 minutes and finally 60 to 100% buffer B in another 15 minutes, all at a flowrate of 0.25mL/min.

Storage of Final Enzyme Extract

The final enzyme extract was treated differently depending on the intended fate of the extract. If it was going to be used for characterisation of the enzyme, such as kinetics and specificity, it was dialysed against Mono Q buffer A and then stored frozen at -20°C. Under these conditions it lost no activity over two months. If the enzyme was going to be used for sequencing, it was dialysed against 0.005% Tween-20 in 50mM ammonium bicarbonate buffer and then freeze dried and stored at -20°C.
4.3. Results and Discussion

An example of a purification schedule, for the purification of 1.6kg of tissue, is given in Table 4.4. The exact amount of purification is not very accurate for two reasons. The first of these is that the tryptophan aminotransferase activity determined in the crude extract also includes the activity of other aminotransferases able to transaminate tryptophan, such as the aspartate aminotransferase. The second is that in the crude extract the aminotransferase activity was very unstable, and tended to disappear completely after an hour at 40°C.

This instability was also apparent in the ammonium sulphate fractionation step, where interference by the ammonium ions occurred. Small amounts of enzyme extract, therefore had to be incubated for long periods to give any idea of activity. However, it can be seen from the purification schedule that this step was extremely productive in removing most of the aspartate aminotransferase activity while retaining most of the tryptophan aminotransferase activity.

The separation given by the Sephacryl 300HR step is shown in Fig. 4.1. The three major UV absorbance peaks were probably due to different groups of compounds. The first was probably due to large protein aggregates, possibly due to the ammonium sulphate precipitate not being dissolved in sufficient buffer, but also partly due to the column being loaded with as much extract as possible; the second peak was due to the extracted proteins. The third peak was the most heavily coloured and so was probably due to the tannins in the extract. As the figure shows there was little separation of the aspartate and tryptophan
<table>
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<td>Phenylsuperose</td>
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<td>7.2</td>
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Table 4.4. Purification of Tryptophan Aminotransferase.
Results of the purification of 1.6 kg of mung beans. A unit is equal to 1 nmol of product produced per minute.
Fig. 4.1. Separation of Tryptophan and Aspartate Aminotransferase Activities on Sephadryl S-300HR.

Conditions were as given in Section 4.2.5: the column was equilibrated with 0.1M NaCl, 10mM tris-HCl, pH 8.0 and was loaded with the sample at 20mL/hr. The sample was eluted at a flowrate of 45mL/hr and collected in 6mL fractions using the same buffer.

The absorbance at 280nm is given by the continuous line. The tryptophan (×) and aspartate (●) aminotransferase activities were determined for various fractions and are linked by the dashed line (---) and the dot-dashed line (--- - ---) respectively.
aminotransferase activities. The enzyme fraction from this column was stable for only a few days at 4°C.

Both the Fastflow Q and the Mono Q columns had the same type of anion exchange packing, differing only in one being a FPLC column. The Fastflow Q was used since the enzyme fraction from the Sephacryl 300HR was still quite coloured, and it was felt that further purification was required for protection of the FPLC column.

The Fastflow Q was run at a higher pH to decrease the amount of salt required to elute the enzyme, although this decreased the resolution of the aspartate and tryptophan aminotransferase activities (Fig. 4.2). Even with this loss in resolution, the two activities were still markedly separated. The enzyme fraction from this column was completely colourless, and stable at 4°C for several weeks. This stability made it possible to combine several extractions to be applied to the Mono Q column, thus decreasing losses.

The Mono Q column nearly fully resolves the aspartate and tryptophan aminotransferase activities (Fig. 4.3), although the enzyme is by no means pure, as can be seen in the SDS gel shown in Fig. 4.4. Once again, enzyme fractions from several Mono Q column runs were combined to be applied to the next column; Phenylsuperose.

All the aspartate aminotransferase activity from the Phenylsuperose column coelutes with the tryptophan aminotransferase activity (Fig. 4.5). However, the amount of aspartate aminotransferase activity is far less than that applied and is probably due to the aspartate activity inherent in the tryptophan aminotransferase. The enzyme was still not fully pure as can be seen in the SDS gel shown in Fig. 4.6; however, it can be identified. The three lanes in the gel are taken across the tryptophan aminotransferase peak from the Phenylsuperose column, and are loaded with roughly equal amounts of activity. The only band in
Fig. 4.2. Separation of Tryptophan and Aspartate Aminotransferase Activities on Fastflow Q.

Conditions were as given in Section 4.2.5: the column was equilibrated with 10mM tris-HCl, pH9.0, the sample was loaded and then the column washed with the same buffer made 0.15M with sodium chloride. The column was eluted with a linear gradient of 0.15 to 0.45M sodium chloride in 10mM tris-HCl, pH9.0 over a volume of 80mL.

The absorbance at 280nm is given by the continuous line. The tryptophan (×) and aspartate (•) aminotransferase activities were determined for various fractions and are linked by the dashed line (---) and the dot-dashed line (---- - ----) respectively.
Fig. 4.3. Separation of Tryptophan and Aspartate Aminotransferase Activities on Mono Q.

Conditions were as given in Section 4.2.5: the Mono Q column was equilibrated with 10mM tris-HCl, 120mM NaCl, pH8.0. The sample was applied onto the column at a flowrate of 1mL/min, and then the column washed with the equilibration buffer for five minutes. The sample was eluted at a flowrate of 0.25mL/min, using a changing gradient of NaCl in tris-HCl, pH8.0; the salt concentration increased from 120mM to 200mM in 24 minutes, then to 250mM in a further 36 minutes.

The absorbance at 280nm is given by the continuous line. The tryptophan (×) and aspartate (•) aminotransferase activities were determined for various fractions and are linked by the dashed line (---) and the dot-dashed line (--- - ---) respectively.
Fig. 4.4. Polyacrylamide Gel Electrophoresis of Mono Q Tryptophan Aminotransferase Fraction.

Four fractions from the Mono Q column were separated by polyacryl-amide gel electrophoresis using the method of Laemlli (1970); the gel was stained with Coomassie blue. The fractions covered the tryptophan aminotransferase activity peak, going from right to left. Each lane contained an equal amount of protein, approximately 5μg. Lane D had virtually no activity, while the activity ratios for lanes C:B:A were approximately 3:2:1.
Fig. 4.5. Separation of Tryptophan and Aspartate Aminotransferase Activities on Phenylsuperose.

Conditions were as given in Section 4.2.5: the Phenylsuperose column was equilibrated with 50mM phosphate, 1.7M ammonium sulphate, pH7.7. The sample was loaded at a flowrate of 0.25mL/min and the column then washed for five minutes with the equilibration buffer. The sample was eluted using a decreasing gradient of ammonium sulphate, dropping from 1.7M to 1.02M in the first 15 minutes, then down to 0.68M in the next 45 minutes and finally to no ammonium sulphate in another fifteen minutes.

The absorbance at 280nm is given by the continuous line. The tryptophan (×) and aspartate (○) aminotransferase activities were determined for various fractions and are linked by the dashed line (---) and the dotted line (-----) respectively.
the gel which remains at a constant density across the three lanes is the one at about 58kDa, which therefore must be the aminotransferase.

The molecular weight determined by gel electrophoresis, of 58kDa, was found under reducing conditions, and so not necessarily the molecular weight of the enzyme. The molecular weight of the native protein was estimated using gel filtration on a Superose-12 column. The results of this size fractionation are shown in Fig.4.7, with the positions of the four molecular markers denoted by the arrows.

These markers were used to determine the molecular weight of the protein as 59kDa, a value almost the same as the weight determined by gel electrophoresis, suggesting that the enzyme exists in monomeric form. To confirm this value, the elution volume of BSA was compared with that of the enzyme on the Sephacryl 300HR column. The elution volume for BSA was approximately 85mL after the void volume, while the elution volume of the aminotransferase was about 90mL. This suggests that the molecular weight is less than that of BSA, agreeing with the figure of 58-59kDa.
Fig. 4.6. Polyacrylamide Gel Electrophoresis of Phenylsuperose Tryptophan Aminotransferase Fractions.

The three fractions from the phenylsuperose column with the most tryptophan aminotransferase activity were separated by polyacrylamide gel electrophoresis using the method of Laemmli (1970), the gel was stained with Coomassie blue. Lanes A, B and C show the separation of the proteins just before the activity peak, at the activity peak and just after the activity peak, respectively. The amount of protein was adjusted in each lane so that the total tryptophan aminotransferase activity was the same, resulting in lane B having approximately half as much protein as lanes A and C. The location of the aminotransferase is marked by the dark arrow.
The image shows a gel electrophoresis result with bands at various mobility values.

- Lane A: Bands at 205 kDa, 97.4 kDa, 66 kDa, and 45 kDa.
- Lane B: Bands at 205 kDa, 116 kDa, 97.4 kDa, and 66 kDa.
- Lane C: Bands at 205 kDa, 116 kDa, 97.4 kDa, 66 kDa, and 45 kDa.

The gel is labeled with molecular weight markers on the left.

- Lane A: Bands at 45 kDa and 29 kDa.
- Lane B: Bands at 45 kDa.
- Lane C: Bands at 45 kDa and 29 kDa.
Fig. 4.7. Resolution of Tryptophan Aminotransferase Activities on Superose-12.

Conditions were as given in Section 4.2.5: the Superose-12 column was equilibrated with 10mM tris-HCl, pH 8.0. The 100μL sample was applied and eluted at a flowrate of 0.4mL/min. In addition a standard mix of catalase (MW 232kDa), β-galactosidase (MW 106kDa), BSA (MW 66kDa) and lysozyme (MW 14.1kDa) were chromatographed under the same conditions, to give the molecular weight markers shown.

The absorbance at 280nm is given by the continuous line; the tryptophan aminotransferase activity is given by the dashed line.
5. CHARACTERISATION OF TRYPTOPHAN AMINOTRANSFERASE

5.1. Introduction

Aminotransferases catalyse fully reversible reactions, thus even if an aminotransferase were solely specific to two substrates and two products, or more correctly four substrates, it would be difficult to assign a role to the enzyme. This is complicated by the low specificity of the enzyme increasing the number of possible substrates for the enzyme. Thus careful characterisation of the enzyme, considering the rates and Michaelis constants for as many of the substrates as possible is required.

The reversible nature of the transamination complicates evaluation of these parameters further. For comparison of the substrate rates, assays should be carried out under the same conditions. An assay exists which makes this possible for comparison of amino acid substrates (Bernt and Bergmeyer, 1967), however it is not very sensitive, and also depends on conditions tending to give equilibrium concentrations of the products. For more accurate determination of substrate rates the assay procedure should involve the removal of one or both of the products, as in the formation of the borate-pyruvate enol complex in the spectrophotometric tryptophan assay (see Section 4.2.2) or the reduction of oxaloacetate in the aspartate assay (see Section 4.2.2). However, these assays use different conditions for each substrate and thus should be compared with care.

In addition to determining the rate of each substrate, the Michaelis constant should also be determined. This gives a measure of the
specificity of the enzyme to the substrate, the lower the $K_m$ the better
the compound is as a substrate. This is important in determining the
principle substrates of the enzyme, but neither the $K_m$ nor the rate of
reaction of various substrates will impart knowledge of the direction of
reaction in vivo.

The effects of various inhibitors on the enzyme are also important.
Inhibitors for aminotransferases fall under several groups. The first of
these is substrate amino acids. If these cause inhibition then the amino
acid is either a substrate with marginal turnover rate, or else substrate
inhibition occurs (Jenkins, 1985)

The second group is carbonyl reagents, such as semicarbazide and
hydroxylamine; these react with the carbon atom of the protonated
internal aldimine (Jenkins et al., 1959). The effects of these inhibitors on
plant aminotransferases are variable depending on the inhibitor used
and sometimes can be reversed by the addition of extra pyridoxal
phosphate (Givan, 1980).

The third group of inhibitors considered were the irreversible
inhibitors iodoacetate and cycloserine. Iodoacetate preferentially
inactivates the pyridoxamine form of the enzyme (Morino et al., 1978),
while cycloserine forms a Schiff base with pyridoxal phosphate within
the catalytic site, inactivating the enzyme. Cycloserine shows different
potencies depending on the type of aminotransferase, being more
effective against alanine aminotransferase than aspartate aminotrans-
ferase (Khomutov et al., 1968).

The use of inhibitor studies will also give some information on the
mechanism of the enzyme in addition to confirming the nature of the
enzyme. Further information on the mechanism can be found by kinetic
studies.
As mentioned earlier (Section 1.4.2) the mechanism for animal aspartate aminotransferases has been elucidated as a ping pong bi bi mechanism (Christen and Metzler, 1985), and this mechanism is the accepted mechanism for plant aspartate aminotransferases (Forest and Wightman, 1973). In the ping pong bi bi mechanism, the enzyme oscillates between two stable forms, for the -aminotransferase this is the pyridoxal form and the pyridoxamine form depending on the state of the cofactor. While in the pyridoxal form, the amino acid substrate binds and is converted to the keto product, with the enzyme converting to the pyridoxamine form. This then binds the keto substrate, which is converted to the amino acid product with the enzyme reverting to the pyridoxal form.

Steady state analysis of this system yields:

\[
\frac{v}{V_{\text{max}}} = \frac{[\text{amino acid}][\text{oxo acid}]}{K_m^{\text{oxo acid}}[\text{amino acid}] + K_m^{\text{amino acid}}[\text{oxo acid}] + [\text{amino acid}][\text{oxo acid}]}
\] (5-1)

which when rearranged to show the amino acid as the varied substrate generates the expression:

\[
\frac{v}{V_{\text{max}}} = \frac{[\text{amino acid}]}{K_m^{\text{amino acid}} + [\text{amino acid}] \left(1 + \frac{K_m^{\text{oxo acid}}}{[\text{oxo acid}]}\right)}
\] (5-2)

Since the $K_m^{\text{amino acid}}$ term in the denominator is not multiplied by any factor, the Lineweaver-Burk plots obtained at different fixed concentrations of the keto acid will be a series of parallel lines. The same results if the amino and keto acids are exchanged. The resulting apparent $K_m$s can be used to find the true $K_m$ for each of the substrates by replotting the apparent $K_m$ against the concentration of the substrate used (Segel, 1975).
Thus it is possible to confirm the identity of the aminotransferase as a tryptophan aminotransferase using various mechanistic, substrate and inhibition experiments. The results of these experiments will feasibly also give some hint as to the role of the enzyme within the cell.
5.2. Materials and Methods

5.2.1. Materials

Chemicals

With the exception of the chemicals given below, all the chemicals used in these experiments were purchased from Sigma Chemical Company at Analar grade.

Acetic acid: AnalaR grade from BDH.
Cycloserine: Analar grade from Nutritional Biochemicals Corp.
Hydrazine hydrate: AnalaR grade from BDH.
Hydroxylamine-HCl: Pronalys grade from May and Baker.
Iodoacetamide: analar grade from Serva.
Mercaptoethanol: Analar grade from Pharmaceuticals ICN.
Potassium and dipotassium phosphate: GPR grade from BDH.
Semicarbazide-HCl: Pronalys grade from May and Baker.

5.2.2. Tryptophan Aminotransferase Preparation

The tryptophan aminotransferase was purified using the procedure given in Section 4.2.5: extraction, ammonium sulphate precipitation, gel filtration, anion exchange chromatography and then hydrophobic interaction chromatography. The enzyme was kept in 10mM Tris-HCL, pH8.0.
5.2.3. Assays

Tryptophan, Aspartate and Tyrosine Aminotransferase Assays

The aspartate and tyrosine assays were the same as those described in Section 4.2.2; the tryptophan assay used was the spectrophotometric assay described in Section 4.2.2.

Phenylalanine and Histidine Aminotransferase Assays

Assay buffer: 0.1M borax, 0.5mM Na$_2$HAsO$_4$, 0.5mM EDTA, pH 8.5.
Amino acid solution: 0.013M phenylalanine or histidine in assay buffer.
2-oxoglutarate solution: 0.13M 2-oxoglutarate in assay buffer.
Pyridoxal phosphate solution: 0.6mM pyridoxal phosphate in assay buffer.

This method is based on the method of Lin and coworkers (1951), stabilising the keto acids formed as an enol complex with borate in the same manner as the tryptophan aminotransferase assay.

The assay was carried out using the same method as the spectrophotometric tryptophan aminotransferase assay (Section 4.2.2), with the exception that the absorbance of the final assay mixtures are read at differing wavelengths. Both the histidine and the phenylalanine aminotransferase assays were read at 310nm. The extinction coefficients used were 9500Lmol$^{-1}$cm$^{-1}$ and 5800Lmol$^{-1}$cm$^{-1}$ for histidine and phenylalanine, respectively.
Alanine Aminotransferase Assay

Assay buffer: 0.05M potassium phosphate, pH7.7.
Alanine solution: 0.06M alanine in assay buffer.
NADH solution: 1mg/mL in assay buffer.
2-Oxoglutarate solution: 0.15M 2-oxoglutarate in assay buffer.
Lactic dehydrogenase: 700U/mL in assay buffer.

The assay used was based on the method of Roswell and coworkers (1972), with the pyruvate formed reduced using lactate dehydrogenase and NADH, and followed by the formation of NAD⁺, in a manner similar to that of the aspartate aminotransferase assay described in Section 4.3.2.

The method of the assay was identical to that of the aspartate aminotransferase given in Section 4.2.2, except with the lactic dehydrogenase replacing the malic dehydrogenase.

Serine Aminotransferase Assay

Assay buffer: 0.05M potassium phosphate, pH7.7.
Serine solution: 0.06M serine in assay buffer.
NADH solution: 1mg/mL in assay buffer.
2-Oxoglutarate solution: 0.15M 2-oxoglutarate in assay buffer.
Glyoxylate reductase: 300U/mL in assay buffer.

The assay used was based on the method of Snell and Walker (1974), with the hydroxypyruvate formed by the transamination of
serine being reduced by glyoxylate reductase with the concomitant oxidation of NADH.

The method was the same as that of the aspartate aminotransferase assay described in Section 4.2.2, with the exception that the reaction showed a moderately high nonspecific loss of NADH, so instead of measuring each cuvette against an air blank and subtracting a blank run, each assay sample was measured against a blank cuvette, containing sample buffer, rather than sample.

Hydroxyphenylpyruvate Aminotransferase Assay

Assay buffer: 0.05M potassium phosphate, pH 7.7.
Glutamate solution: 30mM sodium glutamate in assay buffer.
Ammonium solution: 1.4M NH₄Cl in assay buffer.
NADH solution: 1mg/mL in assay buffer.
Hydroxyphenylpyruvate solution: 7.35mM hydroxyphenylpyruvate in assay buffer.
Glutamic dehydrogenase: 40U/mL glutamic dehydrogenase.

This assay followed the formation of tyrosine and 2-oxoglutarate from glutamate and hydroxyphenylpyruvate. This was done by linking the reaction to the reduction of 2-oxoglutarate to glutamate, using glutamic dehydrogenase:

\[
2\text{-}\text{oxoglutarate} + \text{NADH} + \text{NH}_3 \rightleftharpoons \text{Glutamate} + \text{NAD}^+.
\]  (5-1)

This reaction is reversible; however, at the pH it was performed at, and in the presence of ammonia, the equilibrium lies to the right (Bernt and Bergmeyer, 1967). The amount of ammonia used in the assay was minimised to the level where it still allowed reaction of the 2-
oxoglutarate to occur at a rate sufficiently fast enough not to limit the rate of product formation, but low enough that it did not effect the aminotransferase.

The assay was performed by setting up two cuvettes, the blank and the sample. Both contained 1mL of glutamate solution, 1.7mL of the hydroxyphenylpyruvate solution and 2.5μL of the ammonium solution. To the sample cuvette, 5μL of enzyme solution was added while 5μL of assay buffer was added to the blank cuvette. Then 0.1mL of glutamic dehydrogenase and 0.2mL of NADH was added quickly to the blank cuvette and the sample cuvette in succession, and the reaction monitored at 340nm for twenty minutes. The rate of reaction was determined using the extinction coefficient of 6220Lmol⁻¹cm⁻¹ for NADH.

**Indolepyruvate Aminotransferase**

Assay buffer: 0.05M potassium phosphate, pH 7.7.
Glutamate solution: 30mM sodium glutamate in assay buffer.
Ammonium solution: 1.4M NH₄Cl in assay buffer.
NADH solution: 1mg/mL in assay buffer.
Indolepyruvate solution: 3.5mM indolepyruvate in assay buffer, 0.5mg/mL BHT.
Glutamic dehydrogenase: 40U/mL glutamic dehydrogenase.

This assay was based on the same principle as the hydroxyphenylpyruvate aminotransferase assay, the glutamic dehydrogenase linked oxidation of 2-oxoglutarate. However, since indolepyruvate absorbs strongly at 340nm direct spectroscopy was not possible;
instead fluorospectroscopy was used, following the decrease in fluorescence of NADH. Indolepyruvate still absorbs markedly at the excitation wavelength of NADH, 365nm, but this did not seem to interfere with the assay.

The procedure of the assay was similar to that of the hydroxyphenylpyruvate aminotransferase assay. 1.0mL of glutamate solution, 1.7mL of indolepyruvate solution and 2.5μL of ammonia solution were mixed in a cuvette. Then 20μL of either enzyme solution or assay buffer were added to the cuvette, depending on whether a sample run or a blank run was being undertaken. Then the reaction was started by the addition of 0.1mL of glutamic dehydrogenase and 0.2mL of NADH solution. The change in the fluorescence over twenty minutes was monitored under the conditions of an excitation wavelength of 365nm and an emission wavelength of 425nm.

At the end of the assay period, an known aliquot of NADH solution was added to cuvette and the change in fluorescence measured. The amount of NADH added was calculated to give a change in fluorescence approximately equal to that seen over the time of the assay, but was never less than 50μmol. This standardisation was used to determine the amount of NAD+ formed in both the sample and the blank cuvettes, then the blank was subtracted from the sample to find the true amount of NAD+ formed.

Assaying by Glutamate Formation

Assay buffer: 0.50M potassium phosphate, pH7.7
2-Oxoglutarate solution: 0.13M 2-oxoglutarate in assay buffer.
Amino acid solution: 0.013M amino acid in assay buffer.
Pyridoxal phosphate solution: 0.6mM pyridoxal phosphate in assay buffer.

Glycine-hydrazine buffer: 0.5M glycine, 0.4M hydrazine, adjusted to pH 9.0 with saturated KOH.

Potassium hydroxide solution: 6M KOH.

ADP solution: 33.5mM adenosine-5'-diphosphate in water.

NAD solution: 27mM NAD⁺ in water.

Glutamic dehydrogenase 500U/mL

DNP solution: 0.12mM 2,4-dinitrophenylhydrazine in 35% perchloric acid.

The assay of aminotransferase activity utilising other amino acid donors with 2-oxoglutarate as the oxo donor group was determined by measuring the amount of glutamate formed from 2-oxoglutarate at the end of the incubation period. The method used is based on Bernt and Bergmeyer (1967) as modified by Truelson (1972). The glutamate is measured using the reduction of NAD⁺ associated with the oxidation of glutamate by glutamic dehydrogenase. In this assay, the normal equilibrium of the enzyme reaction (5-1) was shifted towards the formation of NADH and 2-oxoglutarate by trapping the oxo acid with hydrazine, the use of a large excess of NAD⁺ and the alkaline pH.

The initial incubation of the assay mixture was the similar to the method used for the tryptophan aminotransferase assay in Section 3.2.2: 1.0mL of the amino acid solution, 0.1mL of pyridoxal phosphate solution and 0.1mL of the suitably diluted enzyme extract were mixed in a tube and pre-incubated at 37°C for five minutes. The assay was initiated by the addition of 0.1mL of the 2-oxoglutarate solution, and then incubated for three hours at 37°C. This initial incubation of the assay was stopped by the addition of 0.2mL of the DNP solution.
The mixture was then at left -20°C for 15 minutes, before partitioning twice against 5mL of ethylacetate, then twice against 5mL diethylether, to remove excess DNP. The extracted solution was heated on a boiling waterbath to remove the dissolved ether. The pH was adjusted to 9.0 using the potassium hydroxide solution, and the precipitated potassium perchlorate removed by centrifugation. A reaction blank, containing assay buffer instead of enzyme sample was similarly treated.

An aliquot of 0.5mL of this was then added to 2.0mL of glycine-hydrazine buffer, 0.1mL ADP solution and 0.2mL NAD+ solution. The absorbance was measured at 340nm, and the glutamic dehydrogenase was added. After exactly 45 minutes the absorbance was remeasured, and the difference of the two readings used to determine the NADH formed.

For these assays, the specific activity of each of the amino acids was not determined. As an alternative, the amount of reaction was worked out relative to the amount of reaction of tryptophan, thus only yielding relative specific activities.

**Inhibition Assays**

All the inhibition assays were carried out using the spectrophotometric tryptophan aminotransferase assay as described in Section 3.2.2. The putative inhibitive compound was added to the tryptophan solution for ease of dissolving the compound. The amount of inhibition was determined relative to control assays performed at the same time using no inhibitors in the mixture.
5.2.4. Indolepyruvate and Indoleacetic Acid Formation in Cellfree Extracts Supplemented with Tryptophan Aminotransferase.

The procedure for these experiments was exactly the same as the procedure described in Section 2.2.4, with the sole exception that instead of using 0.9mL of crude mung bean extract, 0.8mL of crude extract was used, along with 0.1mL of purified tryptophan aminotransferase sample, containing 50μg of enzyme. The control only contained 0.9mL of boiled crude extract. The final concentration of the various substrates and cofactors used were as given in Table 2.1; the substrates used were: L-tryptophan, pyruvate, NAD+, cocarboxylase, pyridoxal phosphate, and cysteine-HCl.
5.3. Results and Discussion

5.3.1. Specific Activities

The effects of leaving out each of the substrates and the cofactor, pyridoxal phosphate is shown in Table 5.1. As can be seen the effect of omitting either of the substrates is to yield no enzymic activity; the results of the assay with no 2-oxoglutarate proving that the enzyme is not an amino acid oxidase. The effect of omitting the pyridoxal phosphate was only a 5% decrease in activity, showing that the cofactor is not removed during the purification, similar to other plant aminotransferases (Reed and Hess, 1975; Rech and Crouzet, 1974).

The effects of changing pH on the rate of the enzyme was investigated (Fig. 5.1). The enzyme showed the normal broad pH

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</tr>
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<td>5</td>
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<tr>
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</table>

Table 5.1. Assay Requirements Of Tryptophan Aminotransferase.
Fig. 5.1. Effects of pH on the Activity of Tryptophan Aminotransferase.

The effects of pH were determined using the spectrophotometric assay in Section 4.2.2, with the exception that the Assay buffer (100mM borate, 0.5mM Na$_2$HAsO$_4$, 0.5mM EDTA) was adjusted to the various pH's used in the experiment with glacial acetic acid, instead of just using pH8.5 buffer.

Each point on the curve is the average of two assays of the tryptophan aminotransferase activity. All points are from a single enzyme preparation; other preparations gave similar results.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Relative Activity&lt;sup&gt;1&lt;/sup&gt; (Trp = 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>25</td>
</tr>
<tr>
<td>Arginine</td>
<td>35</td>
</tr>
<tr>
<td>Asparagine</td>
<td>5</td>
</tr>
<tr>
<td>Aspartate</td>
<td>5.7</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0</td>
</tr>
<tr>
<td>Glycine</td>
<td>0</td>
</tr>
<tr>
<td>Histidine</td>
<td>4</td>
</tr>
<tr>
<td>Leucine</td>
<td>10</td>
</tr>
<tr>
<td>Lysine</td>
<td>25</td>
</tr>
<tr>
<td>Methionine</td>
<td>0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>75</td>
</tr>
<tr>
<td>Proline</td>
<td>0</td>
</tr>
<tr>
<td>Serine</td>
<td>0</td>
</tr>
<tr>
<td>Threonine</td>
<td>0</td>
</tr>
<tr>
<td>D-Tryptophan</td>
<td>2</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>100</td>
</tr>
<tr>
<td>D-Tyrosine&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>L-Tyrosine&lt;sup&gt;2&lt;/sup&gt;</td>
<td>83</td>
</tr>
<tr>
<td>Valine</td>
<td>3</td>
</tr>
<tr>
<td>No amino acid</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 5.2. Relative Specificity of Protein Amino Acids of Tryptophan Aminotransferase.

1 All amino acids at 10mM final concentration except tyrosine (see 2).
2 Tyrosine final concentration was 5mM, due to solubility; relative activity was compared with a 5mM tryptophan assay.

Optimum associated with aminotransferases (Wink and Hartmann, 1981), although the maxima of about 8.6 is slightly higher than normal maximum of about 8.4 (Forest and Wightman, 1978).
The results of specific activity determinations for the protein amino acids are given in Table 5.2. The enzyme shows a marked specificity towards the L-aromatic amino acids, with the only other amino acids possessing a moderate activity being alanine, arginine and lysine. The enzyme showed practically no activity with the aromatic D amino acids tested.

The activity of various oxo acid acceptor groups with the enzyme are given in Table 5.3. These show that the enzyme has similar activity with 2-oxoglutarate, pyruvate and oxaloacetate, but almost no activity with glyoxylate and hydroxypyruvate.

<table>
<thead>
<tr>
<th>Oxo Acid</th>
<th>Relative Activity$^1$ (2-OG = 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Oxoglutarate</td>
<td>100</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>128</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>116</td>
</tr>
<tr>
<td>Glyoxylate</td>
<td>1</td>
</tr>
<tr>
<td>Hydroxy-pyruvate</td>
<td>9</td>
</tr>
<tr>
<td>No oxo acid</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 5.3. Relative Specific Activity of Oxo Acids of Tryptophan Aminotransferase

$^1$ All oxo acids were at a final concentration of 10mM.
In addition, the rates of the reverse reactions, forming tryptophan and tyrosine, were determined. These reverse reactions were measured using glutamate as the donor amino acid at the same concentration as the oxo acid. The concentration of indolepyruvate used was 1mM due to the instability of aqueous indolepyruvate solutions, and the relative activity compared to tryptophan:2-oxoglutarate at the same concentration. The concentration of hydroxyphenylpyruvate used was 2mM, this was due to the fact that higher concentrations showed some effects of substrate inhibition (see Fig. 5.9b).

The results of the reverse reactions are given in Table 5.4. They show that both reactions occur at a substantially slower rate than that of the forward reaction. These differences do not give an indication of which direction of reaction is the true forward direction of the enzyme in in vivo conditions, since they are not a measure of the equilibrium constant of the reaction, just the rate under the conditions of assay which are markedly different from the normal endogenous situation.

<table>
<thead>
<tr>
<th>Oxo Acid</th>
<th>Concentration</th>
<th>Relative Activity¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>(Trp = 100)</td>
</tr>
<tr>
<td>Indolepyruvate</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>Hydroxyphenylpyruvate</td>
<td>2</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 5.4. Relative Specific Activity of Reverse Reactions of Tryptophan Aminotransferase.

¹ Tryptophan concentrations used for comparison were equal to the concentrations of the oxo acids used.
5.3.2. Michaelis Constants

A summary of the Michaelis constants determined for the major substrates of the enzymes is given in Table 5.5. The graphs used to determine these constants are given in Figures 5.2 to 5.9. The effect of the substrate inhibition by high concentrations of hydroxyphenylpyruvate can clearly be seen in the Lineweaver-Burk plot given in Figure 5.9b. It was not possible to perform evaluations of the Michaelis constants using alanine and aspartate since the assays used would have required excessive amounts of enzyme to give reliable results.

The Michaelis constants for the oxo acids are similar to those of other aminotransferases 0.1 to 1mM (see Table 1.2), although the higher value for 2-oxoglutarate compared to pyruvate and oxaloacetate is

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>0.095</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.07</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.08</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>0.65</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.24</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>0.25</td>
</tr>
<tr>
<td>Indolepyruvate</td>
<td>0.1</td>
</tr>
<tr>
<td>Hydroxyphenylpyruvate</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Table 5.5. Michaelis Constant of Tryptophan Aminotransferase Towards Various Substrates.
Fig. 5.2. Determination of Michaelis Constant of the Aminotransferase Towards Tryptophan.

The Michaelis constant was determined using the direct plot method. The specific activity at each of the tryptophan concentrations was the average of three to five determinations using aromatic amino acid aminotransferase that had been eluted from the Phenylsuperose FPLC column. The method used to assay the enzyme was the spectrophotometric assay described in Section 4.2.2, with tryptophan concentrations altered accordingly. The inset shows a close view of the intersection of the lines to give an improved estimation of the Km value and its error.
Direct Plot of Michaelis Constant for Tryptophan

Enzyme Rate (μ/l/mg protein)

Negative Substrate Concentration (mM)
Fig. 5.3. Determination of Michaelis Constant of the Aminotransferase Towards Phenylalanine.

The Michaelis constant was determined using the direct plot method. The specific activity at each of the phenylalanine concentrations was the average of three to five determinations using aromatic amino acid aminotransferase that had been eluted from the Phenylsuperose FPLC column. The method used to assay the enzyme was the assay described in Section 5.2.3, with phenylalanine concentrations altered accordingly. The inset shows a close view of the intersection of the lines to give an improved estimation of the Km value and its error.
Direct Plot of Michaelis Constant for Phenylalanine

Enzyme Rate (U/mg protein)

Negative Substrate Concentration (mM)
Fig. 5.4. Determination of Michaelis Constant of the Aminotransferase Towards Tyrosine.

The Michaelis constant was determined using the direct plot method. The specific activity at each of the tyrosine concentrations was the average of three to five determinations using aromatic amino acid aminotransferase that had been eluted from the Phenylsuperose FPLC column. The method used to assay the enzyme was the assay described in Section 5.2.3, with tyrosine concentrations altered accordingly. The inset shows a close view of the intersection of the lines to give an improved estimation of the Km value and its error.
The Michaelis constant was determined using the direct plot method. The specific activity at each of the 2-oxoglutarate concentrations was the average of three to five determinations using aromatic amino acid aminotransferase that had been eluted from the Phenylsuperose FPLC column. The method used to assay the enzyme was the assay described in Section 5.2.3, with 2-oxoglutarate concentrations altered accordingly. The inset shows a close view of the intersection of the lines to give an improved estimation of the Km value and its error.
Direct Plot of Michaelis Constant for 2-Oxoglutarate
The Michaelis constant was determined using the direct plot method. The specific activity at each of the pyruvate concentrations was the average of three to five determinations using aromatic amino acid aminotransferase that had been eluted from the Phenylsuperose FPLC column. The method used to assay the enzyme was the assay described in Section 5.2.3, with pyruvate concentrations altered accordingly. The inset shows a close view of the intersection of the lines to give an improved estimation of the Km value and its error.
Direct Plot of Michaelis Constant for Pyruvate
Fig. 5.7. Determination of Michaelis Constant of the Aminotransferase Towards Oxaloacetate.

The Michaelis constant was determined using the direct plot method. The specific activity at each of the oxaloacetate concentrations was the average of three to five determinations using aromatic amino acid aminotransferase that had been eluted from the Phenylsuperose FPLC column. The method used to assay the enzyme was the assay described in Section 5.2.3, with oxaloacetate concentrations altered accordingly. The inset shows a close view of the intersection of the lines to give an improved estimation of the $K_m$ value and its error.
Fig. 5.8 Determination of Michaelis Constant of the Aminotransferase Towards Indolepyruvate.

The Michaelis constant was determined using the direct plot method. The specific activity at each of the indolepyruvate concentrations was the average of three to five determinations using aromatic amino acid aminotransferase that had been eluted from the Phenylsuperose FPLC column. The method used to assay the enzyme was the assay described in Section 5.2.3, with indolepyruvate concentrations altered accordingly. The inset shows a close view of the intersection of the lines to give an improved estimation of the Km value and its error.
Direct Plot of Michaelis Constant for Indolepyruvate
The Michaelis constant (part (a)) was determined using the direct plot method. The specific activity at each of the hydroxyphenylpyruvate concentrations was the average of three to five determinations using aromatic amino acid aminotransferase that had been eluted from the Phenylsuperose FPLC column. The method used to assay the enzyme was the assay described in Section 5.2.3, with hydroxyphenylpyruvate concentrations altered accordingly. The inset shows a close view of the intersection of the lines to give an improved estimation of the Km value and its error.

Part (b) shows the substrate inhibition occurring at 10mM hydroxyphenylpyruvate concentration. The data from part (a) plus the 10mm concentration data are plotted as a Lineweaver-Burk plot, with the inhibition causing the large deviation from linearity of the point closest to the y-axis.
Direct Plot of Michaelis Constant for Hydroxyphenylpyruvate

(a)

(b)
unexpected, since 2-oxoglutarate is the usual oxo acid involved carbon and hydrogen shuttles and so is the most common oxo acid in cells.

The Michaelis constants for the main amino acid substrates are markedly lower than most other aminotransferases, which usually have a $K_m$ of about 0.5 to 5 mM (see Table 1.2). The $K_m$s of the oxo acids in the reverse direction are also quite low. Thus the enzyme is unusually specific for an aminotransferase, suggesting that it has an important role associated in some way with the metabolism of aromatic amino acids.

5.3.3. Inhibition Results

The goals of the inhibition studies were threefold: firstly to discover the inhibitory effects of other amino acids, secondly to investigate the effects of known aminotransferase inhibitors and thirdly to investigate the effects of compounds similar to or involved in indoleacetic acid biosynthesis.

Inhibition by Protein Amino Acids

The amino acids were tested as inhibitors because this is a good measure of whether the amino acid is a substrate with a marginal turnover rate (Jenkins, 1985). The results of amino acids as inhibitors are given in Table 5.6. In most cases the amount of inhibition is approximately related to the specific activity of the enzyme when the amino acid is used as a substrate. In some cases there are large differences, the main ones being histidine, serine and cysteine.
The effect of histidine is probably due to the similarity of structure between histidine and the aromatic amino acids. The Michaelis constant for tryptophan in the presence of 5mM histidine was determined (Fig. 5.10); this gave a $K_m$ of 10mM, and showed that the inhibition was competitive.

The inhibition by serine is probably also due to competitive inhibition, but the high amount of inhibition made it impossible to determine this experimentally. However, the oxo acid derived from serine is hydroxypyruvate, and, as can be seen in Table 5.3, the aminotransferase shows very little activity with hydroxypyruvate.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>45</td>
</tr>
<tr>
<td>Arginine</td>
<td>67</td>
</tr>
<tr>
<td>Asparagine</td>
<td>20</td>
</tr>
<tr>
<td>Aspartate</td>
<td>12</td>
</tr>
<tr>
<td>Cysteine</td>
<td>92</td>
</tr>
<tr>
<td>Glycine</td>
<td>11</td>
</tr>
<tr>
<td>Histidine</td>
<td>65</td>
</tr>
<tr>
<td>Leucine</td>
<td>34</td>
</tr>
<tr>
<td>Lysine</td>
<td>53</td>
</tr>
<tr>
<td>Methionine</td>
<td>64</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>29</td>
</tr>
<tr>
<td>Proline</td>
<td>7</td>
</tr>
<tr>
<td>Serine</td>
<td>93</td>
</tr>
<tr>
<td>Threonine</td>
<td>12</td>
</tr>
<tr>
<td>Valine</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 5.6. Inhibition of Tryptophan Aminotransferase by Protein Amino Acids.

All amino acids were at a concentration of 5mM.
Inhibition by histidine was investigated as described in Section 5.2.3, using a histidine concentration of 5mM in the final assay mixture. The points on the uninhibited tryptophan (■) are those used in Figure 5.2. The specific activity at each of the tryptophan concentrations, when inhibited by histidine, (○) was the average of three determinations using aromatic amino acid aminotransferase that had been eluted from the Phenylsuperose FPLC column.
Reciprocal Tryptophan Aminotransferase Activity
(mg/U)

Reciprocal Substrate Concentration (L/mmol)

-2

0.02

0.04

0.06

Tryptophan Only

Tryptophan & Histidine
hence the inhibition may be due to serine binding in the active site, forming hydroxypyruvate and the reacting very slowly.

The cause of the inhibition by cysteine is unknown and was investigated in the next section.

Aminotransferase Inhibitors

A number of inhibitors towards aminotransferases were tested on the enzyme. Most of these were similar to substrates for aspartate and

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Relative Activity(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With PLP</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>70</td>
</tr>
<tr>
<td>Semicarbazide</td>
<td>100</td>
</tr>
<tr>
<td>Tyrosine-hydrazide</td>
<td>91</td>
</tr>
<tr>
<td>Cycloserine</td>
<td>76</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>74</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>85</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>94</td>
</tr>
</tbody>
</table>

Table 5.7. Effects of Aminotransferase Inhibitors on Tryptophan Aminotransferase.

All inhibitors were at 1mM concentration.
1. Activity relative to an assay under the same conditions without any inhibitors, expressed as percentage of the control.
2. nd = not determined.
alanine aminotransferases, except that instead of undergoing transamination, they reacted with the active site deactivating the enzyme.

The first group of inhibitors were the carbonyl reagents, whose effects are based on the reactivity of the protonated carbon of the internal aldimine (Jenkins et al., 1959), these were hydroxylamine, semicarbazide and tyrosine-hydrazide. As can be seen in Table 5.7, hydroxylamine showed considerable inhibitory activity which was counteracted by supplying pyridoxal phosphate, an effect frequently seen (Givan, 1980). The effects of semicarbazide were similar, but the amount of inhibition observed was much less. The tyrosine-hydrazide was not effected by the presence of pyridoxal phosphate, and its inhibitory effect was low. This is probably due to the fact that it had to compete with the much greater concentration of tryptophan for the active site and the inhibitor should have been tested at a greater concentration.

Cycloserine is an analogue of alanine and inhibits by forming a Schiff base with pyridoxal phosphate. It is highly reactive with alanine aminotransferases, but reacts much more slowly with aspartate aminotransferases (Khomutov et al., 1968). As might be expected with an enzyme even further removed from alanine aminotransferase, cycloserine did not show a great deal of inhibitory activity. In contrast, 0.1mM D-cycloserine completely inhibited the activity of the D-tryptophan aminotransferase from pea plastids (McQueen-Mason and Hamilton, 1989). This difference is startling and suggests that there are major differences between the active sites of the L- and D-amino acid aminotransferases.

Iodoacetate inhibits by reacting with pyridoxamine form of the enzyme (Morino et al., 1978), however the effects vary from one enzyme to another and Gamborg and Wettar (1963) saw little effect on
the aromatic amino acid aminotransferase they extracted. Thus the small amount of inhibition of the enzyme by iodoacetate, as shown in Table 5.7, is not unexpected.

The effects of a reducing agent, mercaptoethanol, and a sulphydryl acetyllating agent, iodoacetamide, were also investigated to try and discover the reason for the inhibition by cysteine. Neither showed inhibitory effects as great as cysteine (Table 5.7), which suggests that the effects of cysteine were due to a competitive mechanism. The amount of inhibition by iodoacetamide was similar to its inhibition of tryptophan aminotransferase from *Streptomyces griseus*, at about ten percent inhibition (Speedie *et al.*, 1975).

**Inhibition by IAA Analogues and Tryptophan Metabolites**

The results of the study of inhibition by indoleacetic acid analogues and tryptophan metabolites in indoleacetic acid metabolism is given in Table 5.8. These show that there is no inhibition by indoleacetic acid, implying that the auxin does not control its synthesis by feedback control on this aminotransferase. However, naphthaleneacetic acid, one of the auxin analogues, does inhibit the enzyme.

Neither tryptamine nor tryptophol, possible precursors in indoleacetic acid biosynthesis, show much effect on the enzyme. Yet serotonin, or 5-hydroxytryptamine, markedly inhibits the enzyme.

The effects of hydroxyphenylpyruvate and indolepyruvate as inhibitors could not be measured due to interference with the assay. However, as mentioned in Section 5.3.1, hydroxyphenylpyruvate did
Table 5.8. Inhibition of Tryptophan Aminotransferase by Indoleacetic Acid Analagues and Tryptophan Metabolites.
All inhibitors were at a concentration of 1mM.
1. Activity is relative to an assay under the same conditions, but with no inhibitor, expressed as a percentage of the control.

show inhibitory effects at higher concentrations, and thus the enzyme may be regulated by feedback inhibition to some extent, although the concentration required, greater than 5mM, is almost certainly beyond normal physiological concentrations.

Indolepyruvate and Indoleacetic Acid Formation in Cellfree Extracts Supplemented with Tryptophan Aminotransferase.

The results of the study of indolepyruvate and indoleacetic acid formation of cellfree extracts supplemented with the tryptophan
aminotransferase are given in Figure 5.11. As can be seen from the figure these results are similar to the results obtained using solely cellfree extract (Fig. 2.3), with no significant amounts of indolepyruvate or indoleacetic acid being formed.

The amount of enzyme added would have been expected to convert approximately 2μmol of tryptophan to indolepyruvate under the assay conditions, but probably less under the conditions of the incubation. However since 1μmol of tryptophan was used in the incubation, it would be expected that a significant amount of the tryptophan would still be converted. This was not the case, which suggests that the major reason for the lack of conversion of tryptophan to indolepyruvate and indoleacetic acid was the breakdown of indolepyruvate after it was formed to various undetected compounds or some form of metabolic channelling usually occurred, but this had been disrupted by the extraction.

5.3.4. Mechanism

The ping pong bi bi mechanism of the aminotransferase was established using kinetic studies. The results of the experiments are given in Figures 5.12 to 5.14. Since the effect of decreasing the substrate concentration for both tryptophan and 2-oxoglutarate is a set of parallel lines in the Lineweaver-Burk plots (Fig. 5.12 and 5.13), the order of the reactants is unimportant, and so the mechanism is the same as that determined for other aminotransferases (Snell, 1985).

Using the above data the true Michaelis constants for the two substrate were also determined (Fig. 5.14). The values determined were 0.1mM for tryptophan and 0.65mM for 2-oxoglutarate.
Fig. 5.11. Formation of Indolepyruvate and Indoleacetic Acid by Cellfree Extracts Supplemented with Tryptophan Aminotransferase.

Each bar on the graph is the average of three measurements of the compound using the same extract, and the error plotted is the standard deviation of the three values divide by the square root of the number of measurements.

The experiment was carried out as described in Section 5.2.4. The incubation buffer used was 50mM phosphate 1mM MnCl₂, 2mM EDTA, 0.1mM pyridoxal phosphate, 1mM pyruvate, 12.5mM NAD⁺, 10mM cocarboxylase and 10mM cysteine-HCl with 150,000DPM [³H]-tryptophan. additionally 50μg of enzyme in 0.1mL was added. The mixture was incubated for an hour at 40°C, then stopped by the addition of glacial acetic acid, followed by 0.1mg of indoleacetic acid and 0.1mg of indolepyruvate. The indoleacetic acid and indolepyruvate were separated and quantitated by Sepralyte C₁₈ and PRP columns as described in 2.2.3 and 2.2.4.
Supplementation with Tryptophan Amino transferase
Conversion of L-Tryptophan in Cellfree Extracts
Fig 5.12. The Effects of Changing 2-Oxoglutarate Concentration on the Tryptophan Michaelis Constant.

The specific activity at each of the 2-oxoglutarate concentrations was the average of three to five determinations using aromatic amino acid aminotransferase that had been eluted from the Phenylsuperose FPLC column. The method used to assay the enzyme was the method described in Section 5.2.3. The 2-oxoglutarate concentrations used to construct the graph were 10mM (■), 1mM (○) and 0.1mM (◆). The straight lines fitted to the points were calculated using least squares linear regression. The data used in this figure was also used in Figure 5.13.
Reciprocal Tryptophan Aminotransferase Activity (mg/U)

Reciprocal Substrate Concentration (1/mmol)

[2-Oxoglutarate] = 10 mM
[2-Oxoglutarate] = 0.1 mM
Fig. 5.13 The Effects of Changing Tryptophan Concentration on 2-Oxoglutamate Michaelis Constant.

The specific activity at each of the tryptophan concentrations was the average of three to five determinations using aromatic amino acid aminotransferase that had been eluted from the Phenylsuperose FPLC column. The method used to assay the enzyme was the method described in Section 5.2.3. The tryptophan concentrations used to construct the graph were 10mM (△), 1mM (●) and 0.1mM (○). The straight lines fitted to the points were calculated using least squares linear regression. The data used in this figure was also used in Figure 5.12.
Reciprocal Tryptophan Aminotransferase Activity
(mg/U)

Reciprocal Substrate Concentration (1/mM)

-2
-1
0
1
2
3
4
5
6
7
8
9
10

0.001
0.002
0.003
0.004
0.005
0.006
0.007

[\text{[Tryptophan]} = 10\text{mM}]

[\text{[Tryptophan]} = 1\text{mM}]

[\text{[Tryptophan]} = 0.1\text{mM}]

\text{\( \Delta \)}}
(a) A direct plot of the apparent 2-oxoglutarate Michaelis constants against the tryptophan concentration at which they were determined. This gives the true $K_m^\text{trp}$ for tryptophan: $K_m^\text{trp}$ amino acid in equations 4-1 and 4-2.

(b) A direct plot of the apparent tryptophan Michaelis constants against the 2-oxoglutarate concentration at which they were determined. This gives the true $K_m^\text{oxo}$ for 2-oxoglutarate: $K_m^\text{oxo}$ acid in equations 4-1 and 4-2.
(a) Replot of 2-Oxoglutarate Michaelis Constants for Varying Tryptophan Concentrations

(b) Replot of Tryptophan Michaelis Constants for Varying 2-Oxoglutarate Concentrations
5.3.5. Identity, Purity and Role of The Tryptophan Aminotransferase.

The results of the characterisation of the enzyme show that it is an aromatic amino acid aminotransferase, able to transaminate pyruvate, 2-oxoglutarate, and oxaloacetate.

Due to the multispecificity of aminotransferases, it is difficult to prove that contaminating aminotransferases have been removed. To try and prove this, the effects of aspartate and alanine on the aminotransferase was investigated, by determining the change in the Michaelis constant. The results are shown in Figures 5.15 and 5.16. Each figure shows that the Km of the enzyme towards tryptophan is increased by the presence of the extra amino acid, but that the maximal rate is unchanged. Thus all the alanine and aspartate aminotransferase activity are due to the same single enzyme, rather than a contaminating enzyme.

Therefore the enzyme is definitely an aromatic amino acid aminotransferase. However the role that the aminotransferase plays in the plant is not known. It may be involved in indoleacetic acid biosynthesis, but it might also be involved in tyrosine and phenylalanine biosynthesis, aromatic amino acid catabolism or alkaloid synthesis.
Inhibition by alanine was investigated as described in Section 5.2.3, using a **alanine** concentration of 5mM in the final assay mixture. The points on the uninhibited tryptophan (■) are those used in Figure 5.2. The specific activity at each of the tryptophan concentrations, when inhibited by alanine, (○) was the average of three determinations using aromatic amino acid aminotransferase that had been eluted from the Phenylsuperose FPLC column.
Reciprocal Tryptophan Aminotransferase Activity
(mg/U)

Reciprocal Substrate Concentration (μmol)

-2

0.01 0.02 0.03 0.04 0.05

Tryptophan Only

Tryptophan & Alanine
Fig. 5.16. Investigation of Inhibition by Aspartate.

Inhibition by aspartate was investigated as described in Section 5.2.3, using a *aspartate* concentration of 5mM in the final assay mixture. The points on the uninhibited tryptophan (■) are those used in Figure 5.2. The specific activity at each of the tryptophan concentrations, when inhibited by aspartate, (○) was the average of three determinations using aromatic amino acid aminotransferase that had been eluted from the Phenylsuperose FPLC column.
6. SEQUENCING

6.1. Introduction

Determination of the primary amino acid sequence of proteins provides valuable information about the protein, including its relationship with similar proteins in the same organism and equivalent proteins in related species. A partial protein sequence can also be used to find the nucleotide sequence of the gene, which can then be used as a tool for measuring changes in the levels of the mRNA for the protein.

The primary sequence of more than thirty aminotransferases are known; the majority of these are animal aspartate aminotransferases (Mehta et al., 1989). However, some plant aspartate aminotransferases and some bacterial and animal tyrosine aminotransferases have been sequenced (Fig. 6.1).

A large amount of sequence similarity is seen between different animal aspartate aminotransferases, with approximately 50% similarity between mitochondrial and cytoplasmic isozymes, and 85% similarity between isozymes in different animals (Graf-Hausner et al., 1982). While the sequence similarity between animal and E. coli aspartate aminotransferases, at approximately 40%, is not as great there is still considerable sequence similarity, with the portions of the sequence showing the greatest divergence being external residues not involved in the active site (Fotheringham et al., 1986).

A comparison of plant aspartate aminotransferases with animal and bacterial aspartate aminotransferases reveals that there is greater sequence similarity between plant and animal sequences, at
approximately 50%, than between plant and bacteria, at approximately 40% sequence similarity (Taniguchi et al., 1992).

Only three aromatic amino acid aminotransferases have been sequenced at this point (Fig. 6.1): the *E. coli* tyrB aminotransferase (Fotheringham et al., 1986) and the rat and human tyrosine aminotransferases (Hargrove et al., 1989; Rettenmeier et al., 1990). The two mammalian sequences show greater than 90% identity, but possess only approximately 40% sequence similarity with the *E. coli* sequence. In addition to the homology between the tyrosine aminotransferases from different species, there is considerable homology between tyrosine and aspartate aminotransferase sequences within a species; for example, there is a 43% homology between the two enzymes in *E. coli* (Fotheringham et al., 1986).

These similarities between sequences suggested that there may be conserved residues or short sequences of amino acids within the sequences. This was investigated by Mehta and coworkers (1989), who showed that there were no large scale similarities over 23 sequences, but that there were twelve amino acids that were conserved over all the sequences examined (see Fig. 6.1). These residues were either involved in the active site or intradomain structure.

Therefore, a partial sequence of the purified aromatic amino acid aminotransferase would give further comparisons between the enzyme and other similar enzymes in other plants, animals and bacteria. In addition, the partial sequence could be used to back translate short oligomeric sections of nucleotides to be used as primers for PCR, thus developing a probe for the detection of the mRNA. This probe could be used to measure changes in the amount of the aromatic amino acid aminotransferase during plant or organ development.
Fig. 6.1. Alignment of Tyrosine and Plant Aspartate Aminotransferase Sequences.

Aminotransferase sequences were arranged to match with the alignments given by Mehta and coworkers (1989) for the alignment of animal and bacterial aminotransferases

# - Conserved Amino acid
+ - Pyridoxal phosphate binding site

Millet mitochondrial aspartate aminotransferase^a
Millet cytoplasmic aspartate aminotransferase^a
Carrot aspartate aminotransferase^b
Alfalfa aspartate aminotransferase^c
Lupin aspartate aminotransferase^d
E. coli tyrosine aminotransferase^e
Rat tyrosine aminotransferase^f
Human tyrosine aminotransferase^g

^a: Taniguchi et al., 1992
^b: Turano et al., 1992
^c: Udvardi and Kahn, 1991
^d: Reynolds PH (pers. comm.)
^e: Fotheringham et al., 1986
^f: Hargrove et al., 1989
^g: Rattenmeier et al., 1990
AMINOTRANSFERASE ALIGNMENTS

1 | MAALSRAASCL | TRRFQMPSRLQQARARAAMA | 29
1 | MA | 2
1 | M | 1
1 | ARENITPS | PTASS | 13
1 | SSSLIPSLSLQQNDKLVGNSLRFSEKQMNFSNASKSRSISMVAVN | 50
1 | MDsyViQTDVDsLSSVLVDVHNI | .GGRNSVQ.GRKkGRkRkARW | 41
1 | MDPYMIQMSKGNLPSILDYLHNV | .GGRSSVP.GKMKGRkRkARW | 41

S...SLFghVepAPKDPILGVTEAYLADPSDKVNVGVG...AYRDN 71
3 SQVASVFAGIAQAFDPDILGVTFVKDNPSVKNLGVG...AYRTE 47
2 SVFANVRAFEDPILGVTVAYHDQSINPKLNLGVG...AYRTE 43
14 D...SVFMVLRAFEDPILGVTVAYKNDPSIKKLNGLGVG...AYRTE 55
51 V...SRFEGIPMAFDPDILGVSEAPROADTSDKLNLGVG...AYRTE 92
1 VFGKVDAAYADPIITLREIKEDPSRPDSDKVNLISG...LYYtED 40
42 D...VRPSDMSkNTenkPRAVDMVQPNKNKTVIDLSIGDPTVFkNLp 88
42 S...VRPSDMSkNTenkPRAVDMVQPNKNKTMLsISIGDPTVFkNLp 88

72 GkPlVLcDVcerea.RiAGNL...NME...YLPmggSvmKAmEESLkLYagEd 116
48 GkPlVLNvVrRaeQ...MlINDSRVKE...YLPitGlaeyNKsAKLIFGAd 94
44 GkPlVLNvVkkAeQ...MLvNdSQSRVE...YLPVglADFKNSAKLIFGAd 90
56 GkPlVLdVcRrRveR...QlLNDSRRKE...YIPVglADFNKSAKLIFGAd 102
41 LiQyViKrvkAENLMlERQ...NKE...YLaIEglaANkFAkELLMGAd 130
42 GkPlVLNvVrRaeE...RSyLYpmeGLNCYrHAIAPLyLFAd 87
89 TDPVeVTQAmkDA...LDSGk...YNg...YAPsYGLSsREEVASYYHCHe 130
89 TDPVeVTQAmkDa...LDSGk...YNg...YAPsYGLSsREEVASYYHCHe 130

117 SeliDKriaAvqAQLStGTAcrLFAdPOkFLPDsQyIyIpTptWsnHHl 166
95 SPlQenRvatVQcLSgTgSLRvGgeFLAHYeHTyIYPVTWgHnpKv 144
91 SPlQenRvatVQcLSgTgSLRvGgeFLAHYeHTyIYPQPTWgHNPKI 144
103 SPlQenRvatVQcLSgTgSLRvGgeFLAHYeHTyIYPQPTWgHNPKI 144
139 NPAkQQRVATVQgLSgTSLRvGAliEYFPgAKvlSAPTWgHnkI 188
88 HPVkQRVRvATQTVGGSAkVgADFkRYtPSGvVVsdPTwEHVAI 137
131 APlEAKDvILTSgsQAsIAEC.LAV.LAN..PGQnLIPRPgFSLyTL 175
131 APlEAKDvILTSgsQAsIAEC.LAV.LAN..PGQnLIPRPgFSLyTL 175

167 WrDAQVPkYAYYYPHESGRLDFAGLMNdIKnApDGsFSfMLhACAHNPtG 216
145 FTLAgLTVSRyYyDpATrgDLFNSGgLdLSSApLGsLVSllhACAHNPtG 194
141 FTLAgLTVSRyYyDpATrgDLFNSGgLdLSSApLGsLVSllhACAHNPtG 190
153 FNLAgLTVSRyYyDpATrgDLFNSGgLdLSSApLGsLVSllhACAHNPtG 202
189 FNDARVPwSeyRyypkTPvGLFdFgSMEdIkAAtAEPGTFTvVLLHGCahNPtG 238
138 FAgAFeFvPYYDDeATNgvFNdllLATkLpARsIVLHtphCnHNPtG 187
176 AEsMgIEvKLnll.Pek.WsIDkQLeIDkETACLvV.NNPswNPCG 222
176 AEsMgIEvKLnll.Pek.WsIDkQLeIDkETACLvV.NNPswNPCG 222
The best ways of monitoring the changes in the amount of aromatic amino acid aminotransferase are to directly measure the aromatic amino acid aminotransferase activity by assay, or determine the amount of the enzyme by antibody techniques. The first method is not feasible, since it is not possible to measure the activity of solely the aromatic amino acid aminotransferase in crude extracts due to the effects of contaminating aminotransferases as discussed in Section 4.3. And since the aromatic amino acid aminotransferase was not purified to homogeneity, it was not possible to develop antibodies against the enzyme.

Thus the approach chosen to attempt to measure the levels of the aminotransferase was aimed at partial sequence analysis to provide information necessary to construct primers for PCR reactions. The product of the PCR reaction would be used to manufacture a DNA probe to follow the changes in the levels of the mRNA. While this approach does not monitor changes in enzyme half life, it was still believed to be viable, since changing mRNA levels are used to regulate enzyme levels in ethylene biosynthesis (Section 1.3.2) and to control tyrosine aminotransferase levels in the liver (Section 1.4.1).
6.2. Materials and Methods

6.2.1. Materials

Chemicals

With the exception of the chemicals given below, all the chemicals used in these experiments were purchased from Sigma Chemical Company at Analal grade.

Acetonitrile: analysed HPLC reagent from JT Baker.
Acrylamide: electrophoresis purity from BioRad.
Ammonium persulphate: Electran grade from BDH.
Chlorofom: Analal grade from BDH.
EDTA: Analal grade from Gibco BRL.
Ethanol: 99.7-100% Analal grade from BDH.
Guanadine isothiocyanate: >97% (AT) from Fluka Biochimika.
Isoamyl alcohol: Analal grade from BDH.
Lithium chloride: Analal grade from BDH.
Methanol: Analal grade from BDH.
Phenol: UltraPure grade from Gibco BRL.
Sodium acetate: Analal grade from BDH.
Sodium citrate: Analal grade from BDH.
Sodium dodecylsulphate: electrophoresis purity from BioRad.
TEMED: Electran grade from BDH.
Trifluoroacetic acid: protein sequencing grade from Sigma.
Trypsin: sequencing grade from Boehringer.
In addition, a Pharmacia QuickPrep mRNA extraction kit was used for the extraction and purification of poly(A) RNA.

**Plant material**

Mung beans (*Vigna radiata*) were purchased from Woolworths Ltd. They were soaked for 16 hours prior to sowing on water saturated vermiculite, and were grown in the dark for six days at 20°C.

**Columns**

The reverse phase column used for HPLC was a Brownlee Aquapore RP-300 C8 column, 2.1mm x 22cm long with a 300nm pore size.

**6.2.2. Gels**

**SDS-Polyacrylamide Gel Electrophoresis**

Polyacrylamide gel electrophoresis was carried out in the manner described in Section 4.2.4. Five well combs were used to allow application of more sample to the gel, and aliquots of 5μg and 10μg bovine serum albumin were electrophoresed separate from, but at the same time as the samples to allow rough estimation of the amount of protein in each band.
6.2.3. Protein Sequencing

Electroblotting onto PVDF Membranes

CAPS buffer: 0.01M 3-[cyclohexylamino]-1-propanesulphonic acid, 10% methanol, pH11.

Ponceau S stain: 0.1% Ponceau S in 1% acetic acid.

Electroblotting was performed immediately after running the SDS gel electrophoresis, before any staining. The gel was equilibrated in CAPS buffer for seven minutes, and then placed against two sheets of PVDF membrane, which had been wetted with methanol; the second sheet was a backing sheet to prevent loss of protein if too much was present to bind to the first sheet. The electroblotting was carried out at 90V/cm for 35 minutes in CAPS buffer.

Protein bands were detected by staining the membrane with Ponceau S stain for one minute, followed by destaining with water. If the protein sample was to be used for *in situ* digestion, it was used immediately, without allowing it to dry. However, if the protein was to be used for N-terminal sequencing, the membrane was dried and stored at -20°C until required. For sequence analysis, the protein bands of interest were excised from the membrane, cut up and placed in a mosaic pattern on the sequencing cartridge of the sequencer.
**In Situ Trypsin Digestion**

PVP solution: 0.5% PVP-40 in 0.6% acetic acid.

Digestion buffer: 100mM Tris pH8.5, 0.5% acetonitrile.

Guanadine hydrochloride solution: 6M guanadine hydrochloride in 0.1% trifluoroacetic acid.

Trypsin: 1μg/μL in 0.01% trifluoroacetic acid, made up on ice immediately before use.

This was performed directly after electroblotting, to prevent excessive absorption of the protein to the PVDF membrane. The bands of interest were cut from the membrane. In addition, several similar sized pieces of the same PVDF membrane to serve as digestion blanks. The membrane pieces were placed into separate eppendorf tubes, to which 1.2mL of PVP solution was added. The tubes were then heated to 37°C for thirty minutes. This treatment was to prevent the trypsin from being absorbed onto the PVDF membrane.

The membrane pieces were washed six times in MilliQ water to remove the excess PVP. Next the membrane bands were cut into fragments no larger than 1mm² and replaced in the same eppendorf tube. The fragments were covered with 50μL of digestion buffer and 1μL of trypsin added. The mixture was left to incubate overnight at 37°C.

After digestion, the tubes were cooled and spun to collect any condensate, the supernatant was transferred to a second eppendorf tube. The membrane fragments were washed with another 50μL of digestion buffer and the wash combined with the supernatant. The membrane fragments were re-extracted with guanadine hydrochloride solution, to remove some of the more strongly bound peptides from the
PVDF. This extraction consisted of adding 50µL guanadine hydrochloride to the fragments, heating at 37°C for thirty minutes, cooling and spinning to collect the condensate and removing the supernatant.

Microbore RP-HPLC

Solvent A: 0.1% trifluoroacetic acid in MilliQ water.
Solvent B: 90% acetonitrile in MilliQ water, 0.085% trifluoroacetic acid.

The HPLC equipment used for separating the tryptic digestion fragments was an Applied Biosystems 140A Solvent Delivery System with a 1000S Diode Array detector. The column used was a Brownlee Aquapore 0.2 x 22cm C8 column.

Prior to each sample run, 10µL of 10% trifluoroacetic acid was added to each of the samples, to acidify the sample. The column was equilibrated with 90% solvent A, 10% solvent B at a flowrate of 200µL/min; 50µL of sample was injected onto the column, followed by another 50µL approximately one minute later. Approximately thirty seconds after the application of the sample, the elution gradient was initiated. This was a 45 minute linear gradient, changing from 10% solvent B to 65% solvent B.

Peptides were detected by absorbance at 214nm, and collected by hand into eppendorf tubes and stored at -20°C until they were used for sequencing. The control tube was run under identical conditions to detect peptides produced from trypsin. A solvent only run was performed between each run to ensure that the column was not retaining any peptides.
Sequencing

N-terminal and internal sequence analysis was performed by Dr David Christie on an Applied Biosystems Model 470A gas phase sequencer equipped with a model 120A on-line phenylthiohydantoin amino acid analyzer. The results were captured using a Model 610A 1.2.1 Information System from Applied Biosystems.

6.2.4. Computer Based Sequence Analysis

Sequence analysis was carried out using the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package (Devereux et al., 1984). The version used was Version 7.1-UNIX, installed in June 1992.

Comparison of the data was performed using two methods: Bestfit and FastA. Bestfit found the optimal alignment of two sequences using the Smith and Waterman local homology algorithm, and was used to find the sequences of greatest homology within various aminotransferase sequences towards the peptide fragments that were sequenced. FastA was used to find the similarity between the peptide fragment sequences and a group of sequences, in this case the SWISS-PROT database, Release 23.0 (8/92). The method uses a hash-table look up search based on the method of Pearson and Lipman (1988).
6.2.5. RNA Extraction

mRNA Extraction

DEPC treated water: 0.1% diethyl pyrocarbamate in MilliQ water, left overnight then autoclaved.

Extraction buffer: buffered aqueous solution of guanadine thiocyanate and N-lauroyl sarcosine.
High salt buffer: 10mM Tris-HCl, pH7.4, 1mM EDTA, 0.5M NaCl.
Low salt buffer: 10mM Tris-HCl, pH7.4, 1mM EDTA, 0.1M NaCl.
Elution buffer: 10mM Tris-HCl, pH7.4, 1mM EDTA.
Glycogen solution: 10mg/mL glycogen in DEPC treated water.
K Acetate solution: 2.5M potassium acetate pH7.4.

Initial attempts to extract mRNA from mung beans was carried out using a Pharmacia QuickPrep mRNA purification kit. Mung beans were grown as described in Section 4.2.5.

The growing tips and primary leaves were harvested directly into liquid nitrogen, and ground in a mortar and pestle, still under liquid nitrogen. Approximately 0.45g of the resulting powder was weighed out and homogenised in 1.5mL of extraction buffer using a Polytron PC2 homogenise from Kinematica GmbH. This homogenate was spun at 12000g for 5 minutes, after the addition of 3mL of elution buffer.

The Oligo(dT) column had been prepared by resuspending the matrix, and then draining the column of liquid by centrifuging at 350g for two minutes with top and bottom closures open. 4mL of the homogenate supernatant was added to the column, and the matrix and supernatant mixed for ten minutes by inversion. The column resin was
separated from the suspension by centrifuging at 350g for two minutes, and then carefully decanting off the supernatant.

The column was then washed in a similar manner, except using high salt buffer three times, followed by low salt buffer once. Finally the poly(A)RNA was eluted using three 0.25mL applications of elution buffer, which had been warmed to 65°C. The successive elutions were collected in a single screw topped microcentrifuge tube; a 50μL sample of this was diluted twofold to be used to measure the concentration of the RNA.

The remaining RNA solution was then precipitated, by adding 50μL of K acetate solution, 10μL of glycogen solution and 1mL of 95% ethanol, chilled to -20°C. The sample was left at -20°C for at least thirty minutes, then the RNA collected by centrifuging for five minutes at 13,000rpm in a microcentrifuge at 4°C. RNA not used immediately was stored in this precipitated state at -70°C. RNA was redissolved in DEPC treated water, with allowance made for the fact that not all RNA redissolves.

RNA Extraction

Denaturing solution: 4M guanadine isothiocyanate, 25mM citrate, pH7.0, 5% N-lauroyl sarcosine.
2M NaOAc: 2M sodium acetate, pH4.0.
Phenol: water saturated phenol.
LiCl: 4M lithium chloride.
3M NaOAc: 3M sodium acetate, pH4.0.
Resuspension buffer: 25mM citrate, 1mM EDTA, pH7.0, 0.5% SDS.
Mung beans were grown as described in Section 4.2.5. The growing tips and primary leaves were harvested directly into liquid nitrogen, and ground with mortar and pestle. Approximately 1g of the resulting powder was added to 5mL of denaturing solution to which 40μL of 2-mercaptoethanol had been added and vortexed. To this mixture 0.5mL of 2M NaOAc, 5mL of phenol and 0.5mL of chloroform was added sequentially, with thorough mixing after each addition. The extract then chilled on ice for fifteen minutes, and then spun at 12,000g for fifteen minutes at 4oC.

The supernatant was recovered and one volume of isopropanol added, the two were mixed and left to precipitate at -20oC for an hour. This was spun at 3000g for ten minutes; the pellet was recovered and vigorously resuspended in 1mL LiCl by vortexing for ten to fifteen minutes and then spun again at 3000g for ten minutes. The pellet was recovered and treated with LiCl in the same manner as previously. This treatment with lithium chloride was to remove polysaccharides, which interfere with isolation of RNA.

The resulting pellet after the second lithium chloride treatment was dissolved in 4mL resuspension buffer, vortexing for five minutes, and then the tubes were left to sit for twenty minutes before vortexing for another five minutes. The resulting solution was extracted with 5mL of chloroform, and the mixture separated by centrifuging at 6000g for ten minutes.

The supernatant was recovered and 1/10 volume of 3M NaOAc followed by one volume of isopropanol were added sequentially with mixing. This was incubated at -20oC for an hour, then the precipitate collected by spinning at 12,000g for twenty minutes. The pellet was recovered and washed with 70% ethanol then pure ethanol, both at -20oC. All remaining ethanol was removed in vacuo, and then the pellet was
redissolved in 100μL DEPC treated water. The presence of RNA was checked by obtaining a spectrum of a one in one hundred dilution of the solution between 200 and 300nm. Poly(A) RNA was purified from the total RNA prepared by this method using the Pharmacia mRNA preparation kit as described in the previous section.
6.3. Results and Discussion

6.3.1. Protein Sequencing

The initial sequencing approach was to attempt to determine the N-terminal sequence of the polypeptide from the blotted sample. Two attempts were made, using approximately 5μg in each case, to sequence the N-terminus; neither attempt gave any sequence data. This suggested that the N-terminus of the polypeptide was blocked. This was not unexpected since the N-terminus of other aminotransferases have been found to be blocked. For example, the N-terminus of the cytoplasmic aspartate aminotransferase of millet is blocked (Taniguchi et al., 1992) and the N-terminus of the rat tyrosine aminotransferase is blocked with an acetyl group (Hargrove et al., 1989).

Therefore the remaining amino acid sequencing attempts were carried out on peptic fragments produced from in situ trysin digests of the protein on the PVDF membrane. The digest was performed on 10 to 15μg of protein. The peptides released directly into the digestion buffer and those extracted using the guanadine hydrochloride were chromatographed on the HPLC separately. The resulting separation traces of the tryptic peptides for each of these samples and their respective blanks are given in Figures 6.2 and 6.3.

The amount of the peptides extracted by guanadine hydrochloride was not sufficient to be used for sequencing, although if the two sets of peptides had been combined prior to HPLC there may have been several fragments in both the samples which, when combined, may have yielded sufficient peptide for sequencing. Three of the peptide
Fig. 6.2. Chromatograph of Peptides Released by *In Situ* Digestion of the Aromatic Amino Acid Aminotransferase.

Conditions were as in Section 6.2.3: the C8 reverse phase column was equilibrated with a 90:10 mixture of 0.1% aqueous trifluoracetic acid:0.085% trifluoracetic acid in 90% aqueous acetonitrile. The sample was injected onto the column in two 50μL aliquots at a flowrate of 0.2mL/min, and the column washed for a short period. The protein fragments were eluted with a linear gradient from 10% 0.085% trifluoroacetic acid in 90% aqueous acetonitrile to 65% 0.085% trifluoroacetic acid in 90% aqueous acetonitrile in 45 minutes.

Part (a) of the figure shows the peptides produced by the overnight trypsin digestion of the small PVDF membrane pieces onto which the tryptophan aminotransferase had been blotted. Part (b) of the figure shows the peptides produced by the overnight trypsin digestion of PVDF membrane pieces which had no protein blotted onto them; this shows the peptide fragments which were formed by the self digestion of trypsin. The peptides labelled rtry10, rtry12 and rtry17 in part (a) were the three fragments that were sequenced.
Fig. 6.3. Chromatograph of Peptides Extracted by Guanadine Hydro-chloride from the In Situ Digestion of the Amino Acid Amino-transferase.

Conditions were as in Section 6.2.3: the C8 reverse phase column was equilibrated with a 90:10 mixture of 0.1% aqueous trifluoracetic acid:0.085% trifluoroacetic acid in 90% aqueous acetonitrile. The sample was injected onto the column in two 50 mL aliquots at a flowrate of 0.2mL/min, and the column washed for a short period. The protein fragments were eluted with a linear gradient from 10% 0.085% trifluoroacetic acid in 90% aqueous acetonitrile to 65% 0.085% trifluoroacetic acid in 90% aqueous acetonitrile in 45 minutes.

Part (a) shows the peptides recovered by re-extraction, using guanadine hydrochloride, of the PVDF membrane pieces after the trypsin digest of the tryptophan aminotransferase. Part (b) shows the peptides recovered from a similar re-extraction of the control pieces of PVDF membrane.
fragments released during the digest were of sufficient quantity to be used for sequencing. These were the fragments labelled rtry10, rtry12 and rtry17 (see Fig. 6.2a).

The three peptides gave the following sequences.

rtry10:
(Val) - Asn - Ala - Leu - Tyr - Ser - Thr - Pro - Ser - ??? - Tyr - Thr

rtry12:
??? - (Leu) - Glu - Val - Ile - (Asp) - Gln - Gly - (Ser) - Asp - (Asp)

rtry17:
??? - ??? - Gly - Pro - Asn - Thr - Asp - Ser - Leu - Ala - Asp - Ala

??? = unidentified amino acid
(???) = uncertain identification of the amino acid.

The sequence fragments were also used to search the SWISS-PROT protein bank to check that the protein was not a different one that had already been sequenced. In all three cases the proteins that gave the best match to the fragments were of higher similarity than the matches of the fragments with the aminotransferase sequences: fragment rtry10 had 50% identity with the yeast chitin synthase, rtry12 had 80% identity with the E. coli phnj protein and rtry17 had 50% identity with the Bacillus NADH dehydrogenase.

None of these three matched proteins were found in the best forty matches of the other two sequence fragments, and indeed no protein in the forty most optimal alignments was found to be common to all three
fragments. Thus the fragments do not come from a single protein that has already been sequenced.

The alignment of each of these fragment sequences to each of the aminotransferase sequences given in Figure 6.1 was performed and the results are shown in Figure 6.4. As can be seen from the figure, fragment rtry12 had little common alignment with all the sequences. However, both rtry10 and rtry17 both matched with common portions of the aspartate and the *E. coli* tyrosine aminotransferase sequences, although not with the mammal tyrosine aminotransferase sequences. The match of both these two sequence fragments were 30 to 40% identity and approximately 60% similarity to the aminotransferase sequences.

These results suggest that the aromatic amino acid aminotransferase has greater homology to the *E. coli* tyrosine aminotransferase than to mammal tyrosine aminotransferases, and also has a fair homology to the plant aspartate aminotransferases.

### 6.3.2. RNA Purification

Both the attempts at directly purifying the mRNA using the Pharmacia preparative kit, and first purifying the total RNA prior to using the kit yielded little or no RNA. The most likely reason for this was that the polysaccharides in the plant cell walls interfered with the purification methods, especially the direct method. This could be seen in the yield of RNA with the total RNA preparation, which was less than 1μg of not very pure RNA per gram of tissue. This was too little to be used for preparation of poly(A)RNA, and due to time constraints the experiments were halted. The problems with extraction of the RNA may
Fig. 6.4. Alignment of Sequenced Fragments of the Aromatic Amino Acid Aminotransferase with Other Aminotransferases.

The sequenced fragments were aligned against the aminotransferase sequences given in Figure 6.1, using the method described in Section 6.2.4.

The three fragments are distinguished as follows:

<table>
<thead>
<tr>
<th>rtry10</th>
<th>(V)NALYSTPS?YT</th>
<th>grey</th>
</tr>
</thead>
<tbody>
<tr>
<td>rtry12</td>
<td>(L)EVI(D)QG(S)D(D)</td>
<td>single box</td>
</tr>
<tr>
<td>rtry17</td>
<td>GPNTDSLADA</td>
<td>double box</td>
</tr>
</tbody>
</table>
have been avoided if a more rigorous method of extraction, such as the hot phenol method (Verwoerd et al., 1989), was used.
7. CONCLUSIONS

The role of the concentration of indoleacetic acid with respect to its effects on plant growth is not fully understood. It has been suggested that sensitivity to indoleacetic acid, rather than its concentration, is the controlling factor in auxin effects (Trevawas 1981, 1982, 1991). However, it is likely that concentration is important, as can be seen in crown gall tissues (Thomashaw et al., 1986) and also in the relationship observed between indoleacetic acid and growth in *Zea mays* mesocotyl (Bandurski et al., 1988).

The route of indoleacetic acid biosynthesis is not yet elucidated. However, there is mounting evidence that indolepyruvate is involved as an intermediate in the formation of indoleacetic acid. Of the other putative intermediates, indoleacetaldoxime is unlikely since it has not been observed in all plants (Cooney and Nonhebel, 1989a), while tryptamine was not converted to indoleacetic acid when formed at very high levels in a transgenic plant (Songstad et al., 1990). In contrast, it has been shown that indolepyruvate was synthesised at a rate compatible with it being the principle, if not sole, intermediate for indoleacetic acid formation (Cooney and Nonhebel, 1991b).

The precursor for indoleacetic acid synthesis has been considered to be L-tryptophan for many years (Erdmann and Schiewer, 1971; Heerkloss and Libbert, 1976). However, there is also evidence that the precursor may be D-tryptophan, produced from L-tryptophan (Law, 1987; Tsurusaki et al., 1990), or that neither D-tryptophan nor L-tryptophan but some compound between anthranil late and tryptophan in the shikimate pathway, possibly indole (Baldi et al., 1991; Wright et al., 1991). Thus while it is probable that indolepyruvate is the
intermediate in the formation of indoleacetic acid. The precursor at the first committed step is still not known.

Depending on the precursor of indoleacetic acid, several different enzymes may be involved in the formation of indolepyruvate. The enzyme studied was that catalysing the conversion of L-tryptophan to indolepyruvate: tryptophan aminotransferase. This was chosen since L-tryptophan is the best documented precursor to indoleacetic acid, and also since changes in the activity of the enzyme had been associated with changes in auxin dependence of tobacco tissue cultures (Gaal and Koves, 1981).

An enzyme with L-tryptophan aminotransferase activity was purified 33,600 fold from mung bean shoot tips and primary leaves. Characterisation of this enzyme showed it to be an L-aromatic amino acid aminotransferase, using pyruvate, oxaloacetate and 2-oxoglutarate as keto group donors. The enzyme had K_m's of 0.07, 0.095 and 0.08mM for phenylalanine, tryptophan and tyrosine, respectively and 0.65, 0.25 and 0.24mM for 2-oxoglutarate, oxaloacetate and pyruvate, respectively.

This was the first purification of a plant aminotransferase which has maximal specific activity with tryptophan (see Table 4.1). The K_m values for the enzyme are also lower than other tryptophan aminotransferases, by between a factor of four (Truelson, 1972) and several hundred (Forest and Wightman, 1973). Thus the enzyme is both more specific to and more active with tryptophan than any others purified previously.

The enzyme was similar to other plant aminotransferases in several respects. It had only a marginal requirement for the cofactor pyridoxal phosphate; the molecular weight, at 58 kDa, was similar to one group of plant aminotransferases; and inhibition by carbonyl reagents followed a similar pattern to that of other aminotransferases.
A partial amino acid sequence of the enzyme was also performed. This produced three fragments that showed similarity to both plant aspartate aminotransferases and to bacterial tyrosine aminotransferase sequences, suggesting that the enzyme is part of the same evolutionary family (Mehta et al., 1989).

The role that the aromatic amino acid aminotransferase plays in indolepyruvate formation and control of indoleacetic acid synthesis can only be surmised. It is not subject to feedback inhibition by indoleacetic acid, so feedback control may be excluded. While the specificity for tryptophan is much greater than other aminotransferases towards their substrates, the $K_m$ is still higher than the expected tryptophan concentration of 1-15μM (Gilchrist and Kosuge, 1980), and this combined with the inhibitory effect of other amino acids mean that the aromatic amino acid aminotransferase would be converting tryptophan to indolepyruvate at a rate much lower than its maximal rate.

This latter effect may well be modified by the effects of compartmentation, this could be an important facet in maintaining the step down in concentration from tryptophan to indolepyruvate.

Control of the activity of animal aminotransferases seems to be carried out by controlling the amounts of the enzyme itself (Hargrove and Granner, 1980). However, since it was not possible to purify the enzyme to homogeneity and form antibodies to it nor to obtain a probe to the enzyme mRNA, it was not possible to see whether this was the case with the aromatic amino acid aminotransferase.

Attempts to synthesize indoleacetic acid from both D-tryptophan and L-tryptophan in cellfree extracts resulted in no significant formation of indolepyruvate or indoleacetic acid compared to a control. This result was also obtained when the cellfree extract was supplemented with a purified extract of the aromatic amino acid.
aminotransferase. This failure to form indoleacetic acid was probably due to the breakdown of indolepyruvate prior to formation of indoleacetic acid combined with the dilution of enzymes due to the lysis of the compartment in which they were contained. Thus the cell-free extract incubations did not yield any information on the roles of tryptophan and indolepyruvate in the biosynthesis of indoleacetic acid.

Indoleacetic acid and indolepyruvate concentrations in mung bean hypocotyl suspension cultures were measured using GC-MS SIM. The indoleacetic acid levels were scattered but mostly remained at moderately low levels throughout the growth of the suspension culture, with no observable relationship between the development of the culture and the concentration of indoleacetic acid.

In contrast, the indolepyruvate levels increased initially and then maintained a maximum concentration. This corresponded with the growth rate of the suspension culture, with the increase in indolepyruvate concentration matching the increasing growth rate before the logarithmic growth phase of the culture was attained, and the maximum level of indolepyruvate occurring during the logarithmic growth phase.

While the indoleacetic acid concentrations did not show any observable correlation to growth or to the indolepyruvate concentrations, the levels of both indoleacetic acid and indolepyruvate were maintained at similar concentrations. In most cases the difference in concentration between indoleacetic acid and indolepyruvate was less than a factor of two.

Hence it is possible that there is a link between the concentration of indolepyruvate and that of indoleacetic acid. This is further supported by the implication of the incorporation of deuterium into indolepyruvate and indoleacetic acid at rates compatible with the former being a
intermediate in the formation of the latter (Cooney and Nonhebel, 1991). The presence of this link suggests that a major portion of the control of indoleacetic acid levels is in the step or steps prior to indolepyruvate synthesis, but that further control of indoleacetic acid concentration occurs, either in the conversion of indolepyruvate to indoleacetic acid, indoleethanol reduction and oxidation, conjugate synthesis and hydrolysis or in indoleacetic acid catabolism.

Thus the results of these experiments in this study further implicate the role of indolepyruvate as an intermediate in the biosynthesis of indoleacetic acid. The role of the aromatic amino acid aminotransferase in indolepyruvate synthesis and hence indoleacetic acid biosynthesis was studied by purification and characterisation of the enzyme, but no conclusive results were produced.
FUTURE EXPERIMENTS

The quantification of indoleacetic acid and indolepyruvate concentrations in suspension cultures should be repeated. In addition, they should also be carried out in auxin-habituated cultures, and possibly also crown gall cultures to see if any change in the indolepyruvate concentration pattern occurs.

Further attempts should be made to fully purify the aromatic amino acid aminotransferase and develop antibodies against it, in order to monitor changes in amounts of the enzyme with development of plants or cultures and compare with changes in indolepyruvate concentrations.

The sequencing of the aromatic amino acid aminotransferase should be completed, preferably using a cDNA library and a probe against the mRNA generated, so that the changes in the level of the mRNA for the aminotransferase can be studied in concert with the changes in the concentration of both the indolepyruvate and the enzyme itself.
APPENDIX A - CULTURE MEDIUM

The medium used for both callus and suspension cultures was the Gamborg B5 Medium (Gamborg et al., 1968) with 2mg/mL 2,4-D and 0.1mg/mL kinetin. The final concentrations of the various components of the medium are given in Table A.1.

The medium was made up by mixing together stock solutions of macro compounds, micro compounds, vitamins, EDTA, FeSO₄ and hormones, making the volume up to almost one litre, adding 20g of sucrose and, if applicable, 8g of agar, then adjusting the pH to 5.5. The volume was adjusted to one litre and then autoclaved at 121°C for fifteen minutes. The components within the various stock solutions are given in Table A.2.
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>20,000</td>
</tr>
<tr>
<td>KNO₃</td>
<td>2,500</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>250</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>150</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>150</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>134</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>3</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>2</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.25</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>CoCl₂·2H₂O</td>
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</tr>
<tr>
<td>KI</td>
<td>0.75</td>
</tr>
<tr>
<td>FeSO₄</td>
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</tr>
<tr>
<td>Na₂EDTA</td>
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</tr>
<tr>
<td>MnSO₄</td>
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</tr>
<tr>
<td>myo-inositol</td>
<td>1.0</td>
</tr>
<tr>
<td>pyridoxine-HCl</td>
<td>1.0</td>
</tr>
<tr>
<td>nicotinic acid</td>
<td>1.0</td>
</tr>
<tr>
<td>thiamine-HCl</td>
<td>1.0</td>
</tr>
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</table>

Table A.1. Concentrations of Components of B5 Medium.
<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>Strength</th>
<th>Volume mL</th>
<th>Component</th>
<th>Amount g</th>
</tr>
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<tr>
<td>Macro</td>
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<td>KNO₃</td>
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<td></td>
<td></td>
<td></td>
<td>CaCl₂.2H₂O</td>
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</tr>
<tr>
<td></td>
<td></td>
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<td>MgSO₄.7H₂O</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(NH₄)₂SO₄</td>
<td>1.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NaH₂PO₄.2H₂O</td>
<td>1.5</td>
</tr>
<tr>
<td>Micro</td>
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<td>500</td>
<td>KI</td>
<td>0.375</td>
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</tr>
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<td></td>
<td>CuSO₄.5H₂O</td>
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</tr>
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<td></td>
<td></td>
<td></td>
<td>CoCl₂.6H₂O</td>
<td>0.0125</td>
</tr>
<tr>
<td>Vitamins</td>
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<td><em>myo</em>-inositol</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>nicotinic acid</td>
<td>0.025</td>
</tr>
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<td></td>
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<td></td>
<td>pyridoxine-HCl</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>thiamine-HCl</td>
<td>0.25</td>
</tr>
<tr>
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<td>50</td>
<td>2,4-D</td>
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<tr>
<td>Kinetin</td>
<td>100x</td>
<td>100</td>
<td>kinetin</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table A.2. Constituents, Amounts and Strengths of Stock Solutions for Medium Preparation.
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